BERGEIM

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STUDIES ON SALIVARY DIGESTION

BY

OLAF BERGEIM

Ph.G. South Dakota State College of Agriculture and Mechanic Arts, 1906
B. S. South Dakota State College of Agriculture and Mechanic Arts, 1908

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Olaf Bergeim

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IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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in Physiological Chemistry.

P. B. Hawk.

Instructor in Charge

APPROVED: Duncan

acting HEAD OF DEPARTMENT OF Chemistry.

STUDIES IN SALIVARY DIGESTION

INTRODUCTION

The observation that saliva possessed the power of transforming starch to sugar was first made by Leuchs in 1831.(1) Since that time a very large amount of work has been carried out by many able investigators,bearing more or less directly on the relation of the salivary secretion to the digestive processes. It would,therefore,at first appear that little of importance could be added to our knowledge of these relations. We find,however,that many points are still unsettled. We note that much of the older work on the subject is not trustworthy because reliable methods were not available at the time such work was carried out. We further see that many old opinions must be revised and many results re-interpreted in the light of more recent discoveries. Nor can we be said,even yet to be in a position to definitely settle certain important questions.

As to the relative importance of the functions possessed by the salivary secretion,much diversity of opinion has existed and still exists. Its properties of assisting in masticating food and in deglutition are obvious and have,we believe,never been disputed. Some early investigators considered that its only offices were mechanical. Budge(2) came to such a conclusion after extirpating the salivary glands of a rabbit and finding no disturbance of digestion. Bunge(3) was one of the most active supporters of this view and pointed out that some of the cetacea which live in the water possessed no salivary glands. He also refers to the work of Fehr,who removed the salivary glands from dogs and merely found

that the animals drank more water. Bernard(4) studied the secretion in horses after esophagostomy and found that its amount depended rather on the dryness than on the character of the food,the parotid saliva moistening the food and the submaxillary lubricating it. He also pointed out that some animals which do not masticate their food possess submaxillary but only rudimentary parotid glands.

This idea of saliva as a purely mechanical aid was opposed by Lehmann(5) and by Wright(6) who were convinced that it possessed some importance as a digestive agent.The theory is no longer tenable. Among the other passive properties attributed to saliva is that it aids in the preservation of the teeth,clears the mucus membrane, modifies thirst,favors the expression of the voice,as a solvent assists taste,and by aeration assists in the gastric digestion of milk,a more spongy and digestible curd being formed. M.Roger(7) claims that it aids the passage of substances lodged in the esophagus,not only by its viscosity,but by its excitation of the pharynx causing movements of swallowing and a peristaltic wave.

The relation of the salivary to the gastric secretion has been the subject of several investigations. Biernacki(8), Wulfson(9) and Wright(6) pointed out that food mixed with saliva gave rise to a greater secretion than the food alone. Frouin(10) claimed that there was an increase in the quantity,acidity,and digestive power with a resulting greater rapidity of digestion and evacuation. A solution of sodium carbonate of the same alkalinity had little effect. Lehmann(5) on the other hand made experiments with the aid of gastric fistulas and found no more secretion with saliva than with other mucus foods,and considered such an action as hardly necessary,nitrogenous foods commonly

ingested giving rise to much greater gastric secretion than such saliva. Hemmeter(11) confirmed this work for dogs after extirpation of the salivary glands. He claims that the salivary glands secrete internally a secretin which,carried by the blood to the stomach there causes a flow of gastric juice.

Among others Roger(7) claims that saliva also acts as a preventive of hyperacidity in the stomach. He introduced acidulated water into the stomachs of dogs and cats and by esophageal fistulas observed that there was an abundant flow of saliva,the secretion stopping when the stomach contents were neutralized.

In early childhood,according to Allaria(12),saliva ,on account of its low osmotic pressure,may play an important part in reducing any tendency toward a hypre-osmotic condition in the stomach. Among the minor properties credited to the salivary secretion are those of splitting certain glucosides and peptonizing fibrin(Roger) and of splitting peptides(Koelker,13). It also contains small amounts of maltase and invertase. The peptolytic properties are probably due to traces of erepsin-like ferments found distributed thruout the body,and are not specific.Aside from its functions in the body,according to Boldini(14),Lisbonne(15) and Wolff and Fernbach(17) salivary amylase possesses the property in common with other amylases of coagulating starch solutions under certain conditions. This is attributed by them to a distinct enzyme,amylo-coagulase.

By far the most important constituent of human saliva and the one with which we are mainly concerned here is its amylolytic enzyme. Until recently there was a tendency on the part of most investigators to discount the part taken by this enzyme

in the digestion of starchy foods,in spite of the fact that it has been repeatedly shown that saliva possesses a very strong amylolytic power,and that considerable amounts of sugars and dextrin have often been observed in the contents of human stomachs. This was partly due to conflicting views brot about by studies on dogs in the saliva of which there is usually no amylase. The main contention was,however, that salivary digestion must cease soon after the food reached the stomach,as it was known that amylases ceased to perform their work and were even destroyed in the presence of acidities as great as that of the gastric juice. This theory was,however based upon a mistaken idea as to the nature of the stomach movements. As early as 1881 the view that there were two stages of stomach digestion,a stage of amylolytic and a stage of protein digestion was advanced and supported by Chittenden and Griswold(18),the first suggestion having been made by Reinhardt(19) who claimed that the acidity of the gastric juice was low in the first few hours of digestion.

It is only recently,however,that definite knowledge of the digestive processes in the stomach has been obtained. Our present views are based upon the work of Hofmeister and Schütz(20), Moritz(21),Cannon(22),Roux and Balthazard(23),and others. Moritz measured the pressures in various parts of the stomachs of dogs and men and found them to be very slight in the fundic portion. Cannon,and Roux and Balthazard used the Röngten rays to advantage in following the course of digestion in the stomachs of men and lower animals . Grützner fed animals various colored foods and(24) observed their stratification in the stomach. The details of this extensive work cannot be given here,but the results obtained by these different methods of study are in entire agreement. They

show that the stomach as a digestive organ consists essentially of two distinct parts,a pyloric portion which by strong peristaltic waves moving in the direction of the duodenum,thoroly churns and mixes the food with the gastric juice,forcing the contents into the duodenum at intervals;and a fundic portion which does not at all take part in these movements but acts as a reservoir from which the pyloric portion is supplied,and the food contained in which may remain for hours unmixed with the acid gastric juice. The food last swallowed is forced into the center of this mass and is thus also protected from premature mixing. The importance of these facts from the standpoint of salivary digestion can hardly be overestimated,as they make it evident that the salivary amylase may exert its action for several hours under favorable conditions. When we realize that starchy foods make up the greater part of the ordinary diet,that from 600 to 1000 cc.of saliva per day are secreted by adults,and that saliva,as shown by our own experiments,may produce as much as ten times its weight of maltose in thirty minutes,it is no longer possible to consider the main functions of saliva as mechanical.

Another consideration of importance in this connection is that of the adaptation of the salivary secretion to diet. Neilson and associates made considerable study of this question(38) and found that the amylolytic and maltose splitting powers of the saliva uniformly increased on a carbohydrate diet and decreased on a protein diet from the values for a mixed diet. They account for this by an increase in the concentration of the salivary amylase or in its activity. The same conclusion is reached by L.G.Simon(25) who found that the quantity of saliva secreted was, as expected,greatest with dry food,and also that its activity was

greatest on a diet of starchy food. Similarly he claims that the invertin content of saliva increases increases on sucking cane sugar,this increase being lost more or less completely in half an hour. Other work tending to support the possibility of such adaptation is that of Pawlow and associates(26),Walter(27), Wassilief(28) and Lintwarew(29) who showed that the amount and quality of the pancreatic juice of the dog depended on the character of the food. Hirata(30) found 300 to 500 times as much amylase in the pancreas of bread fed rats as in the pancreas of lard fed rats. Ellinger and Cohn(31) give similar results for man. On the other hand we find the apparently conclusive work of Carlson and Crittenden(32) who noted no increase in the ptyalin concentration of human saliva on prolonged vegetarian diet and could find no correlation between such concentration and the diet. They also point out that the amylolytic power of the saliva of monkeys is no greater than that of man,while in some herbivora as goats,sheep and horses it is entirely lacking. Mendel and associates(33) repeated Neilson's experiments with some modifications and could find no adaptation to diet.Wohlgemuth(77) who has done much important work on diastases gives similar results. It is evident that no final decision on the question of salivary adaptation can yet be made altho the evidence against such adaptation seems to be somewhat the stronger.

The more we go into the literature of the subject the more we become convinced of the truth of Lehmann's statement(5) made over 60 years ago:" All experiments and results bearing on the matter of salivary digestion must be adopted with the greatest caution,for there is no analytical inquiry,in which,under apparently precisely similar relations,the same experiments

so often yield different results and in which quantitative determinations so invariably present a want of uniformity".
We have,of course to-day a better knowledge of the precautions necessary for the obtaining of reliable results than was available at that time.We have come to recognize the great influence of slight variations in temperature and the amount of electrolyte on enzyme action. We are also equipped with more accurate methods for the quantitative study of their action. Nevertheless the difficulties still met with in the elimination of all unknown factors,are very great,and trustworthy data can only be obtained by a liberal use of control tests and rigid duplication of conditions in any tests meant to be comparative. One of the principal reasons for this is found in the great variability of the enzyme concentration of the saliva at different times and with different individuals.Some authors have found the daily variations to be uniform. For example Hoffbauer(35) showed that the diastatic power rose from morning till noon and that there was a depression after each meal depending in extent upon the quantity and consistency but not the chemical composition of the food. Schüle(36) obtained similar results and the work has been largely confirmed by Wohlgemuth(77) and by Chittenden and Richards(37),who also showed that the composition of the saliva depended upon the stimulus employed,ether and chloroform,for example,giving a more viscid saliva than was obtained by mechanical stimulation. As might be expected,however,from a secretion which is a mixture eliminated from four different sets of glands, saliva collected at the same time each day from the same individual,will never be uniform enough to be used as identical in quantitative experiments. It may be preserved fairly well under

toluol and such a method will give better results than the use of separately collected samples of saliva,but is not entirely satisfactory. To be strictly comparable however all of the tests of a series must be run simultaneously,and this method of procedure we have observed in all of our experiments.

Further details and discussion relating to these studies are given under their respective headings. The work has been grouped as follows:

I. On the effect of dilution on the rate of hydrolysis of starch by salivary amylase.

II. On certain substances in softened water and in the University water supply which inhibit the action of salivary and pancreatic amylase.

III. On the purification of pancreatic and salivary amylase.

IV. On the reactivation of human saliva which has been made inactive by the action of heat or of acid,and including some tests on the activation of dog saliva.

V. On the iodine-starch reaction at low temperatures.

Part 1

The Effect of Dilution on the Rate of Hydrolysis of Starch by Salivary Amylase

Many investigations have been made on the influence of variations in the concentration of enzymes,of their substrates,and and of electrolytes,on the rate of the reaction.Very little work has,however been done directly on the effect of simultaneous decreases of all three of these,that is,of dilution.For example in the case of the amylases,as was first shown by Kjeldahl(39),the rate of hydrolysis of starch is directly proportional to the amount of enzyme as long as the substrate is present in considerable excess,or until the digestion has proceeded from 30 to 40% of the way to completion.After this point is reached the rate becomes gradually slower,assuming the form of a logarithmic curve,but no expression for the rate of the reaction has been yet proposed which holds true in all cases,probably on account of the variety of factors which influence this rate.As to the effect of varying the concentration of the substrate,the rate of hydrolysis seems to be directly proportional to the amount of substrate as long as the enzyme concentration is not too small compared with that of the substrate,after which the change is practically the same irrespective of the amount of substrate.For electrolytes which may favor the reaction it has been shown that the optimum concentration depends largely upon the concentration of the enzyme and the substrate ,but these remaining constant,the rate of hydrolysis will decrease if the concentration of the electrolyte is shifted in either direction from the optimum,there being of course no law for the rate of this change which will hold for all electrolytes.

In the case of dilution,properly so called,i.e.where only the amount of water is varied,the matter is more complicated and becomes still more so if the diluent itself contains ingredients which affect the rate of the reaction.It seems obvious that what the effect of the dilution will be must depend upon the initial concentration of the enzyme,substrate,and electrolytes. For example,if the enzyme and substrate are present in high concentrations dilution will increase the rate of the hydrolysis by decreasing the concentration of the products of the reaction.

Bradley(40),in his work on lipase found that when water was present to the extent of 50% of the mixture of lipase and ester,the amount of reversion was negligible,and the reaction proceeded to complete hydrolysis of the ester.In the case of starch the reaction ordinarily proceeds to 80 to 85% maltose, apparently reaching an equilibrium.The reaction can be brot to completion by removing the maltose formed by fermentation or by dialysis.This is not necessarily equivalent to dilution,as in the latter case the products of the reaction are not removed but merely diluted and we must consider at the same time the effect of the simultaneous dilution of enzyme and substrate and electrolytes.If a salt such as NaCl or an acid such as HCl is present in a quantity such as to inhibit the reaction,dilution will tend to decrease this inhibition to an optimum point,after which further dilution will tend to decrease the rate of hydrolysis. This is particularly true because,as Paschutin(41) has shown,if neutralyzed saliva is diluted with water in varying proportions,a content of acid,which in undiluted saliva would cause a diminution of diastatic action,in the more dilute solutions actually aids the diastatic process.On the other hand Kübel(42) has shown that the

more concentrated the starch solution the less inhibition will be caused by a given amount of NaCl and the more of this salt will be required for optimum conditions.

From these considerations it will be apparent that whether dilution increases or decreases the action in a given case will depend upon which of the component factors predominates.On account of the complexity of the problem prediction from theoretical considerations is not very safe and the only logical way to arrive at a decision is by systematic experimentation.The object of the present study was to carry out some such experiments upon salivary amylase.

In the older editions of Fischer's "Physiology of Alimentation",page 1o4,occurs the following statement:- "It has been shown by the work of Cannon that the saliva does not act best in the concentration in which it is poured out upon the food,but when diluted with about three times its bulk of water.The explanation of this fact lies no doubt in the dilution of the products of amylolytic activity,for in a concentrated solution the point at which the reaction comes to a standstill is reached sooner than in a more dilute one." This statement,however,as Fischer acknowledges in a private communication,was based on an incorrect interpretation of Cannon's work.There has therefore,as far as our knowledge goes, been no previous experimental study of this problem.

In these experiments the action of several different diluting fluids was tested.Distilled water was used to determine the effect of a simultaneous decrease of the concentrations of all of the constituents of the mixture. A solution of 0.3% NaCl and 0.02% disodium phosphate was employed to determine the effect of

decreasing the concentration of enzyme and substrate while maintaining an optimum electrolyte concentration.Tap water was also used for comparison and two softened waters prepared by mixing one part of lime water and five parts of tap water was tested in some cases.One of these,designated as "3-11" had remained loosely stoppered for several months,while the one designated as "9-11" was prepared shortly before beginning these experiments.Kahlbaum's soluble starch was used as the most suitable substrate,the viscosity of its pastes not increasing as rapidly with the concentration as do pastes prepared from ordinary starch.Starch pastes were always made up on the day of the experiment as starch pastes freshly prepared are much more readily digested than those which have undergone the progressive changes that Maquenne and Roux(43) have termed retrogradation and coagulation. The saliva employed was obtained by chewing pure paraffin to stimulate the flow.Fresh, filtered saliva was used in all cases.

The Effect of Dilution on the Time of Digestion of Starch to the Achromic Point with Iodine

Method:- Into each of a series of Erlenmeyer flasks, of Jena glass and carefully cleaned, varying in size from fifty to one thousand cubic centimeters, were introduced by means of a pipette ten cubic centimeters of a 1% starch paste, made from Kahlbaum's soluble starch. Varying amounts of various waters were then added and the contents were well mixed. Then to each was added 1 cc. of a 10% solution of saliva in that particular water, this being added at thirty second intervals. The contents were thoroly mixed and allowed to stand at room temperature, being covered with watch glasses to prevent evaporation. At intervals a drop of the mixture was removed by means of a pipette and tested on a test tab with a very dilute iodine solution. The end point was taken where the mixture first ceased to alter the pale yellow color of the iodine solution. All readings were corrected for the difference of time in adding the enzyme solution.

Table 1

Diluent: Boiled Distilled Water Total Volume of Mixture:	Temperature 27° C. Time to the Achromic Point:
11.6 cc.	14.0 min.
20.0 cc.	19.5 min.
30.0 cc.	28.0 min.
40.0 cc.	50.5 min.
50.0 cc.	1 h. 6.0 min.
70.0 cc.	1 h. 27.5 min.
100.0 cc.	2 h. 7.0 min.
150.0 cc.	6 h. 30.0 min.
200.0 cc.	9 h. 0.0 min

Table 1 continued

Total Volume of Mixture:	Time to the Achromic Point:
300.0 cc.	14 h. 0.0 min.
500.0 cc.	40 h. 0.0 min.

Table 2

Diluent: 0.3% NaCl, 0.02% Na_2HPO_4 Temperature: 27.0° C.

Total Volume of Mixture:	Time to the Achromic Point
11.0 cc.	---
20.0 cc.	9.0 min.
30.0 cc.	10.0 min.
40.0 cc.	11.0 min.
50.0 cc.	11.0 min.
70.0 cc.	11.0 min.
100.0 cc.	11.0 min.
150.0 cc.	11.0 min.
200.0 cc.	11.0 min.
300.0 cc.	16.0 min.
500.0 cc.	17.0 min.

Table 3

Diluent; Soft Water (3-11) Temperature 20.0° C.

Total Volume of Mixture:	Time to the Achromic Point:
10.0 cc.	2 h. 38 min.
20.0 cc.	3 h. 52 min.
30.0 cc.	3 h. 52 min.
40.0 cc.	3 h. 36 min.
50.0 cc.	3 h. 36 min.
70.0 cc.	4 h. 50 min.
100.0 cc.	4 h. 50 min.

Table 3 continued

Total Volume of Mixture:	Time to the Achromic Point:
150.0 cc.	4 h. 50 min.
200.0 cc.	6 h. 4 min.
300.0 cc.	8 h. 0 min.
500.0 cc.	9 h. 0 min.

Table 4

Diluent: Recently Softened Water Temperature 17-20° C.

Total Volume of Mixture:	Time to the Achromic Point:
11.0 cc.	2 hours
20.0 cc.	6 hours
30.0 cc.	10 hours
40.0 cc.	10 hours
50.0 cc.	12 hours
70.0 cc.	15 hours
100.0 cc.	19 hours
150.0 cc.	21 hours
200.0 cc.	25 hours
300.0 cc.	30 hours
500.0 cc.	30 hours

It will be noted from these tables and from the curves plotted using these data(see curve sheet 1) :-

1. Dilution with distilled gradually increased the time of digestion for saliva until in a 1:5000 dilution very little action takes place.This is the effect that we should expect from the change in the electrolyte concentration,which gradually decreases.

2. Dilution with a sodium chloride-sodium di-hydrogen

phosphate solution of approximately the optimum concentration for salivary digestion,by maintaining the electrolyte concentration has very slight effect on the time of hydrolysis of the starch up to a dilution of 1:5000.

3. Tap water softened by the addition of one fifth of its volume of lime water and allowed to stand for several months, evidently contains substances inhibiting the action of the salivary amylase so that the hydrolysis is considerably delayed. It does,however contain the electrolytes necessary for the enzyme action,this fact tending to counteract the inhibition,so that in dilute solutions (1:1500 and over) the action is more rapid than with distilled water which lacks the electrolyte but contains no inhibiting substance.

4. The freshly prepared softened water evidently contains a considerably larger amount of the inhibiting substance,so that even in dilutions of 1:300 it requires ten hours to reach the achromic point compared with half an hour for distilled water and ten minutes with the chloride-phosphate solution.

The Effect of Dilution with Different Waters on the Amount of Reducing Sugar Produced by a Given Amount of Salivary Amylase in a Given Time

Method:- The study of the relative speeds of hydrolysis of starch by enzymes in varying concentrations of starch and enzyme introduce many difficulties in the way of securing strictly comparative data from experiments made at different times. Noticeable variations may be brot about by small changes in the amylolytic activity of the saliva,by very slight variations of temperature during hydrolysis,or in the determination of the reducing sugar.Also bacterial action is never entirely eliminated. For these reasons it is desirable to run at one time a series complete enough to cover the entire significant portion of the curve of the rate of hydrolysis.The following method was used to eliminate the sources of error as far as possible.

Thoroly cleaned Jena Erlenmeyer flasks of 200 cc.capacity and of the same shape,so as to expose equal surfaces of the contents to the air in the copper reduction method,were used thruout. Two grams of Kahlbaum's soluble starch in the form of pastes of concentrations from two to twenty per cent in the particular water which was to be tested,were introduced.All starch pastes were made up immediately before use,this being particularly important with the more concentrated pastes in which some form of aggregation with cloudiness takes place on standing for several hours,this being accompanied by an increase in the time required for hydrolysis.In order to maintain the relative amounts of starch and enzyme,without increasing the total volume of the mixture above 100 cc.,which would interfere with the reducing sugar determination,the amounts of starch and saliva were gradually

decreased keeping the total volume constant.The flasks were then placed in a constant temperature bath until they had acquired the temperature of the bath,and the saliva solution,previously warmed to the same temperature ,was added from calibrated pipettes at 30 second intervals.In no case was the actual volume of the saliva solution added less than one cubic centimeter,so that appreciable errors in measurements were avoided.After remaining in the bath for 30 minutes the action was immediately stopped by the addition of Fehling's solution,allowance being of course made for the differences in time of starting the determinations.They were then transferred to a vigorously boiling water bath,the surface of the water in the bath coming to the level of the liquid within the flasks,and kept there for fifteen minutes.The reduced cuprous oxide was immediately filtered on Gooch crucibles containing a quarter inch layer of asbestos specially treated with nitric acid, caustic potash and Fehling's solution. The cuprous oxide was thorolywashed with hot water,then with alcohol and ether,and weighed after drying to constant weight in an air bath at 110° C. The above method for determining the amount of reducing sugar is adapted for the special purpose from that given by Sherman,Kendall, and Clark for the determination of diastatic activity.(44)

In some cases the modification of the copper iodide titration method as recently proposed by Kendall was used.(45) In these cases the Gooch crucible was placed on a filter bottle and the cuprous oxide dissolved in hot nitric acid,the asbestos then being thoroly washed with distilled water. This copper nitrate solution was transferred to the original 200 cc. flask, 5 cc.of a sodium hypochlorite solution added and the solution allowed to stand a few minutes. Then 10 cc. of a 5% solution of

phenol were quickly added from a pipette with the tip removed,to combine with the free chlorine.The mixture was neutralyzed with sodium hydroxide solution and then slightly acidified with acetic acid.Finally 10 cc. of a 30% solution of potassium iodide was added and the excess of iodine titrated with standard sodium thiosulphate which had been checked against pure copper and standardized against potassium bichromate solution from time to time.

We have found this method to give good results if the precautions suggested by Kendall are observed.Somewhat less time is required than for the gravimetric determination.In very dilute solutions some difficulty is met with in reading the exact end point as the starch iodine color re-appears on standing a short time.

Peters(46) has recently published a careful study of the sources of error of the copper iodide method which contains many valuable suggestions.

Table 5

Diluent:Soft Water(3-11),9 1/2 months old.

Time: 30 minutes Temperature: 40° C.

No.	No.cc. Saliva	Amount of Starch Paste	Water to make Vol:	No.cc.of $Na_2S_2O_3$	Mgs.of Copper	Mgs.of Maltose	Dilution 1:
1	0.63cc	10cc of 20%	11 cc	131.10	745.4	693.2	16
2	0.63cc	25cc of 8%	26 cc	100.98	559.1	520.0	40
3	0.63cc	25cc of 8%	35 cc	75.30	400.4	354.5	56
4	0.63cc	50cc of 4%	51 cc	49.10	251.6	222.3	80
5	0.63cc	100cc of 2%	101cc	20.75	90.1	78.5	160
6	0.31cc	50cc of 2%	100cc	5.25	46.0	39.0	320
7	0.06cc	10cc of 2%	100cc	.44	29.2	24.0	1600
8	0.03cc	5cc of 2%	100cc	.20	16.0	12.2	3200
9	0.00cc	25cc of 8%	100cc	10.45	64.5	41.8	-----
10	0.00cc	100cc of 2%	100cc	6.15	38.0	24.6	-----
11	0.00cc	5cc of 2%	100cc	.12	.7	.5	-----

In the sodium thiosulphate column the actual amounts used in the titrations are given,while the copper and maltose were calculated after correcting for control tests.

Table 6

Effect of Dilution

Diluent: Tap Water Time 25 minutes Temperature 24° C.

No.	No.cc. Saliva	Amount of Starch Paste	Water to Make Vol:	No.cc.of $Na_2S_2O_3$	Mgs.of Copper	Mgs.of Maltose	Dilution 1:
1	0.5	10cc of 10%	11 cc	73.10	405.7	359.3	20
2	0.5	"	20 cc	72.30	400.9	355.0	40
3	0.5	"	60 cc	55.90	303.5	268.4	120
4	0.5	"	100cc	55.10	296.8	262.4	200
5	0.25	5cc of 10%	100cc	21.10	218.2	192.6	400
6	0.20	4cc of 10%	100cc	14.00	175.3	154.5	500
7	0.10	2cc of 10%	100cc	6.65	164.7	145.0	1000
8	0.05	1cc of 10%	100cc	3.08	149.5	131.4	2000
9	0.00	10cc of 10%	100cc	6.03	36.5		

In the sodium thiosulphate column actual amounts used in the titrations are given,while the copper and maltose are calculated after correcting for check and putting all tests on the basis of one gram of starch.

Table 7

Effect of Dilution

Diluent: 0.3% NaCl,0.02% Na_2HPO_4 Time:30 minutes Temperature 40° C.

No.	Amount of Saliva	Amount of Starch Paste	Water to Make Vol:	No.Mgs. Cu_2O	No.Mgs. Maltose	Dilution
1	0.04 cc	10cc of 20%	11 cc	304.5	251.7	275
2	0.04 cc	"	16 cc	319.8	264.3	400
3	0.04 cc	25cc of 8%	26 cc	373.3	293.4	650
4	0.04 cc	"	36 cc	383.4	301.4	900
5	0.04 cc	50cc of 4%	51.cc	397.0	312.1	1275
6	0.04 cc	100cc of 2%	101 cc	405.9	335.9	2525

Table 8

Effects of Dilution

Diluent: 0.3% NaCl, 0.02% Na_2HPO_4 Time: 30 minutes Temperature: 40° C.

No.	Amount of Saliva	Amount of Starch Paste	Water to Make Vol:	No.Mgs. Cu_2O	No.Mgs. Maltose	Dilution 1:
1	0.1 cc	10cc of 20%	11 cc	289.2	227.1	110
2	0.1 cc	"	16 cc	463.2	364.4	160
3	0.1 cc	25cc of 8%	36 cc	492.8	387.7	360
4	0.1 cc	50cc of 4%	51 cc	492.8	387.7	510
5	0.05cc	50cc of 2%	100 cc	504.2	399.1	2000

Table 9

Effects of Dilution

No.	No.cc. Saliva	Amount of Starch Paste	Water to Make Vol:	No.cc.of $Na_2S_2O_3$	Mgs.of Copper	Mgs.of Maltose	Dilution 1:
1	0.1	10cc of 10%	11 cc	32.60	226.2	198.8	110
2	0.1	"	21 cc	31.72	222.0	195.2	210
3	0.1	"	36 cc	34.10	238.7	210.8	360
4	0.1	"	61 cc	33.11	231.8	204.7	610
5	0.1	"	101 cc	33.22	232.5	205.3	1010
6	0.02	2cc of 10%	100 cc	6.50	225.8	199.4	5000
7	0.01	1cc of 10%	100 cc	3.22	225.4	199.1	10000
8	0.00	10cc of 10%	100 cc	7.10			
9	0.00	2cc of 10%	100 cc	3.50			
10	0.00	1cc of 10%	100 cc	3.05			

All columns are corrected for controls and to the basis of one gram of starch.

Conclusions with regard to the effects of dilution with different waters on the digestion of starch by salivary amylase in more dilute solutions;a discussion of the results given in tables 5,6,7, 8,and 9,and curves 2,3,4,and 5 based on this data

From table number 5 and curve number 2 corresponding to it we see how pronounced an inhibition is exerted on the action of salivary amylase by softened water even after it had stood in contact with the air for several months with the consequent absorption of carbon dioxide.The activity is ten times as great where only 11cc of liquid are used as it is where 100cc of this water have been added and 17 times as great as where 200 cc have been used.From this point on the decrease is not so rapid as the enzyme was not apparently destroyed,but the amount of starch hydrolysis brot about is very small indeed compared with that which occurs under favorable conditions.

From table 6 and the corresponding curve,number 3,which deal with the action of tap water,we see that this also is a case where the salivary enzyme is forced to act under unfavorable conditions other than that of mere dilution.These conditions were however ,by no means so adverse as in the case of softened water, altho the alkalinity to phenolphthalein is greater .This was due partly perhaps to the protecting influence of the large amounts of carbonic acid contained in this water,partly to the lack of the strongly inhibiting hydroxides of the alkaline earths,and also undoubtedly as is indicated by the very fair rate of hydrolysis in the more dilute solutions,to the favoring action of some of the electrolytes contained in this water which are removed by a lime treatment.It will be noticed that in that part of the curve where

the inhibition is practically at its maximum,i.e. in the lower part, the activity in the case of tap water is three to five times as great as with the softened water,altho,for reasons before stated the two curves may be considered comparable only in an approximate way.Neither was their such a pronounced inhibition in the upper part of the curve . Tap water can not,however be considered a very favorable medium for the action of salivary amylase as the action is many times slower than in a sodium chloride solution of favorable concentration.The form of the curve of hydrolysis is such as we should expect from the action of substances which give a reaction unfavorable but somewhat less so than in the case of softened water.

In curves 4 and 5 corresponding to tables 7,8,and 9,the action of a 0.3% sodium chloride solution is considered,in the first two cases there also being present small amounts of disodium phosphate.The most striking fact brot out by this data is that in dilutions of from 600 to 2000 and in the last case even in dilutions as large as 600 to 10000 the curve for the rate of hydrolysis is practically a straight line.This would tend to show that about the optimum concentration of salts were used,and further that when such is the case very large dilutions do not appreciably affect the rate of hydrolysis. On the other hand in the early part of the curve the rate is considerably reduced,due mainly in this case to the high viscosity of the solution,altho in the later stages there would doubtless also be an inhibition due to the accumulation of the products of the reaction. In the case where the sodium chloride was used alone we find that while the medium is favorable the maximum hydrolysis does not lie in such great dilutions,which supports work done by various investigators and

repeated in some of our own ,to the effect that small amounts of di-sodium phosphate will increase the rate of hydrolysis over that for sodium chloride alone.

Optimum Dilutions and the Effect of Dilution with Different Waters upon the Speed of Hydrolysis of Starch by Saliva in More Concentrated Mixtures

In the foregoing series of experiments it will be noted that the amounts of saliva are small compared with the amounts of starch and with the total volumes of the solutions.It will also be noted that in nearly all cases the rate of digestion decreases with the increase in dilution,the only apparent exception being in the cases of dilution with a sodium chloride solution in high starch concentrations,where the limiting factor is quite probably the high viscosities of the solutions.In other words little study was made of the changes of velocity of digestion in the neighborhood of the optimum concentration.Under the conditions of the experiments this was indeed impossible,because no accurate work can be done in high concentrations of saliva where the starch is digested to the achromic point almost instantaneously.The The study of the digestion in these concentrations was,however, considered to be important as approaching normal conditions of salivary digestion and also to find out how far the inhibiting action of some of these waters extended. The reduction of temperature was employed as the method of slowing the reaction without introducing unknown factors. The following preliminary test was made,the time taken to reach the achromic point being measured.

The digestions were carried on in test tubes stoppered and placed in an ice bath,starch paste and saliva both being at 0° C. at the time of mixing.The saliva was added at 30 second intervals and the time readings corrected for this.Some difficulty was found in reading the end point,as explained later,for which reason the time readings are probably a little high.

Table 10

Effects of Dilution in Concentrated Mixtures

Preliminary test

Diluent: Distilled Water Temperature 0° C. End point: Achromic

Amount of 10% Starch Paste	Amount of Saliva	Amount of Water	Dilution 1:	Time to the Achromic Point
5 cc	5 cc	---	2	25 minutes
5 cc	5 cc	5 cc	3	20 minutes
2 cc	2 cc	6 cc	5	16 "
1 cc	1 cc	8 cc	10	11 "
0.5 cc	0.5 cc	9 cc	20	11.5 "
0.1 cc	0.1 cc	9.8 cc	100	16 "

This preliminary test brot out the fact that digestion took place quite rapidly even at zero degrees and that the optimum dilution was in the neighborhood of ten.

This test also pointed out the greatest probable sources of error in a study of this kind as being: 1st,the accurate measurement of the time of digestion;2nd,in having both starch paste and saliva at zero degrees at time of mixing; and 3rd,in the immediate and intimate mixing of the two. To eliminate these errors as far as possible,the following method was used.

Method:- Thoroly cleaned,rubber stoppered,hard glass test-tubes

of 25 cc.capacity were used.They were immersed to within one-fourth inch of the top in an ice mixture . Varying amounts of freshly prepared starch paste were then introduced and an amount of water which,with the saliva to be added later,would make the desired volume. They were well mixed and when the mixture had come to a constant temperature the required amount of saliva,likewise cooled to 0°C.,was added from cold calibrated pipettes at exactly 30 second intervals. The tubes were shaken and immediately put back in the ice mixture. A guide test was run by means of the iodine color reaction and the digestions were stopped before they had proceeded more than about 20% of the way to completion.They were stopped by running in at 30 second intervals,in the same order as before ,5cc. of Fehling's solution.In some cases the whole process was carried out in a cold room(10°C.) to still further obviate the danger of a rise in the temperature of the saliva while being transferred.

When the digestions were completed the contents of the tubes were transferred to 200 cc. Erlenmeyer flasks with the aid of 25 cc. of distilled water,50 cc. of Fehling's solution added, and the flasks placed in a boiling water bath for fifteen minutes. The cuprous oxide was then filtered off,dissolved in nitric acid, and titrated as usual by the iodine method.

Table 11

Effects of Dilution in Concentrated Mixtures

Diluent: Distilled water Time:11.5 minutes Temperature; 0° C.

No.	Amount of Starch Paste	Amount of Saliva	Amount of Water	No.cc.of $Na_2S_2O_3$	Mgs.of Maltose	Mgs.of Copper	Dilution
1	5cc of 10%	5 cc	---	31.90	171.1	193.0	2
2	"	5 cc	5 cc	33.60	179.3	203.3	3
3	2cc of 10%	2 cc	10 cc	35.13	187.5	212.5	7
4	1cc of 10%	1 cc	8 cc	33.70	179.8	203.9	10
5	"	1 cc	13 cc	30.25	161.2	183.0	15
6	0.5cc of 10%	0.5cc	9 cc	28.50	151.8	172.4	20
7	5cc of 10%	---	5 cc	2.10			

All columns corrected for control test number 7.

Table 12

Effect of Dilution in Concentrated Mixtures

Diluent:Filtered Tap Water Time of Digestion:10 min. Temp.: 0° C.

No.	Amount of Starch Paste	Amount of Saliva	Amount of Water	No.Mgs. Cu_2O	No.Mgs. Maltose	Dilution
1	10cc of 10%	10 cc	---	427.6	378.6	2
2	7cc of 10%	7 cc	6 cc	505.7	441.8	3
3	4cc of 10%	4 cc	12 cc	514.2	448.6	5
4	3cc of 10%	3 cc	14.cc	526.6	458.5	7
5	2cc of 10%	2 cc	16 cc	515.0	449.3	10
6	1.5cc of 10%	1.5 cc	17 cc	466.6	410.5	13.3
7	1.3cc of 10%	1.3 cc	17.4cc	363.1	321.5	15.4
8	1cc of 10%	1 cc	18.0cc	345.0	305.4	20
9	0.6cc of 10%	0.6 cc	18.8cc	333.3	294.9	35
10	0.4cc of 10%	0.4 cc	19.2cc	320.0	283.0	50
11	0.2cc of 10%	0.2 cc	19.6cc	325.0	287.6	100
12	10cc of 10%	---	10 cc	24.5		
13	0.2cc of 10%	---	19.8cc	12.6		
14	0.4cc of 10%	---	19.6cc	13.6		

All columns are corrected for check and calculated to the basis of one gram of starch.

Table 13

Effect of Dilution in Concentrated Mixtures

Diluent: 0.3% NaCl Solution Time of Digestion: 9 minutes

Temperature: 0° C. Carried out in room at 10° C.

No.	No.cc.of 10% Starch	No.cc. Saliva	No.cc.of NaCl Sol.	No.cc.of $Na_2S_2O_3$	Mgs.of Copper	Mgs.of Maltose	Dilution 1:
1a	10.0	10.0	---	112.32	513.6	455.0	2
1b	10.0	10.0	---	111.92			
1c	10.0	----	10.0	9.40			
2a	5.0	5.0	10.0	64.95	588.1	521.0	4
2b	5.0	5.0	10.0	65.07			
2c	5.0	---	15.0	6.10			
3a	3.0	3.0	14.0	36.95	557.5	494.7	7
3c	3.0	---	17.0	5.50			
4a	2.0	2.0	16.0	26.61	555.25	491.9	10
4b	2.0	2.0	16.0	25.55			
4c	2.0	---	18.0	3.87			
5a	1.3	1.3	17.4	18.45	556.65	493.1	15
5c	1.3	---	18.7	3.58			
6a	1.0	1.0	18.0	14.42	546.0	483.7	20
6b	1.0	1.0	18.0	14.42			
6c	1.0	---	19.0	3.50			

The duplicates were averaged and corrected for the conlrol test which was run in each case. From these values the copper and maltose columns were calculated.

Conclusions with regard to Optimum Dilutions and the Effect of Dilution in Concentrated Mixtures on the Speed of Hydrolysis of Starch by Saliva; a discussion of results given in tables 10,11, 12,and 13,and corresponding curve sheets 6,7,and 8

From the data on distilled water,tables 10 and 11 and curve number 6 corresponding ,we see that the effect of dilution was gradual and not very marked in extent. The most rapid hydrolysis takes place in about seven dilutions. However the activity is only about 5% less in three dilutions and ten dilutions respectively and beyond ten dilutions it falls off only very gradually due to the gradual decrease in the electrolyte concentration.

Where tap water was used (table 12 and curve number 7) we again find the optimum to be in the immediate neighborhood of seven dilutions,with but slight variation from 5 to 10 dilutions. Beyond this,however,the falling off is much more rapid than in the case of distilled water,indicating the presence of inhibiting substances in this water.The greater activity at the optimum over that at two dilutions must be credited to decreases in the viscosity of the solution and in the concentration of the products of the reaction.

In the case where a dilute sodium chloride solution was used (table 13 and curve sheet number 8),we find as usual a greater production of reducing sugar than where other solutions were employed .The form of the curve of the rate of the reaction is also slightly different.The optimum conditions were found at four dilutions instead of seven as in the two previous cases,the sodium chloride evidently assisting the action in these solutions of high viscosity.On the other hand we find that the hydrolysis

is greater in twenty dilutions than in two or three dilutions and the falling off with the dilution is more gradual,so that all in all the sodium chloride solution is much the most satisfactory medium.

No attempt has been made in these tests to duplicate physiological conditions which are themselves variable. Neither is it claimed that the results obtained can be applied directly and in all cases to salivary digestion within the organism. The work may however give general indications of value and experiments made under controlled conditions must be considered in any attempt at the understanding of the influences which affect the digestive processes as they normally occur.

In this connection it is of importance to know what really happens to ingested water when it reaches the stomach. Liquid alone,as Grützner(24) and others have shown will remain but a short time in the stomach if the intestine is not already full. Best and Cohnheim(90) have shown that the time water remains in the stomach depends very little on its temperature,but somewhat upon the quantity ingested,while a sodium chloride solution remains somewhat longer than water. The same appears,in general,to be true even if the stomach does contain food. Grützner makes the following statement in the summary of his well known studies along this line:-"Mässiges Getränk wahrend der Mahlzeit stört sicherlich die Thätigkeit des gesunden Magens in keiner weise,wie man vielfach angenommen hat". He also cites the work of Leconte(47) who fed two dogs normally,two hours later gave one of them water,and fifteen minutes later examined the stomach contents of both animals. He found scarcely any differences between the two,the water having largely left the stomach and even the duodenum. It

had carried only small particles of food along with it. The water apparently left the stomach along the small curvature without disturbing the food to any extent.

Kaufmann(48) even claims to have demonstrated the presence of a trough on the smaller curvature,after physostigmine injection along which water might readily leave the stomach. Scheunert(49) disagrees with this finding after numerous experiments on horses and dogs,in which the course of the water thru the stomach was followed by means of colored stains contained in the liquid.This author found no evidence of a trough altho a large part of the water did leave along the smaller curvature. To a greater or less extent the water poured over the entire contents of the stomach. He believes that the food may absorb water until it reaches the most desirable consistency,after which no more will ordinarily remain. However,as Grützner has suggested,if the stomach is filled with liquid or semi-liquid food,water may intermix to some extent altho probably only when the stomach empties,as he observed a lasting layer formation even with semi-liquid foods.

In our experiments it was shown that where a 10% starch paste was used as a basis and an equal amount of saliva added, that dilution with from two to five times their combined volume of water(the mixtures then containing from1/2 to 2 1/2% starch) considerably increased the amount of digestion. Further if the electrolyte concentration is kept fairly high(as we should expect upon any ordinary diet),and providing no strongly inhibiting substances are present in the water(which can of course be guarded against),then the rapidity of digestion for the greatest dilutions which could possibly occur in the body would be greater than in

this original mixture of 10% starch paste and saliva. We must consider also the fact that the ordinary starchy foods,such as bread and potatoes,have starch concentrations considerably above 10%,and the above cited facts with regard to the passage of water thru the stomach.

It would appear,that as far as salivary digestion is concerned,there is no danger of excessive dilution with any water of fair quality.

On the other hand it seems probable that a dilution as great as that of the optimum for salivary digestion is not reached under ordinary conditions.For this reason the ingestion of large amounts of water would increase the efficiency of salivary amylase thru the production of a satisfactory dilution of the digestion mixture.

These results are in accord with work carried out in this laboratory showing a better utilization of carbohydrates during periods of high water ingestion.(50)

On the Inhibitory Action of Certain Substances in Tap and Softened Waters with Reference Particularly to Salivary and Pancreatic Amylase

In the previous experiments on the effect of dilution on the speed of salivary digestion,it was constantly noted that the tap water,and especially the softened waters used,exerted a pronounced inhibitory action,the action being greatest with the recently softened water.The following series may be taken as an example.

Table 14

Comparative Tests on the Inhibitory Action of Certain Waters

No.	No.cc.of 2% Starch	No.cc. Water	Kind of Water	No.cc,of Saliva	Time to the Achromic Point
1	50	50	Distilled	0.1	2 hours 5 min.
2	50	50	Soft (3-11)	0.1	25 "
3	50	50	Soft (9-11)	0.1	30 "
4	50	50	Tap Water	0.1	7 "
5	50	50	0.3% NaCl	0.1	35 min.
6	50	50	0.3% NaCl with 0.5gm.Na Benz.	0.1	35 min.

The sodium benzoate was added merely to compare the inhibitory action of a large amount of antiseptic with that of the small amounts of inhibiting agents present in the water.As was to be expected it was negligible.

To still further test the inhibitory action of these waters, to determine the relative inhibition with smaller amounts of water;and especially to compare the inhibition of the action of salivary amylase with that of pancreatic amylase the four series of tests given in tables 15 and 16 were made.

Table 15
Effects of Various Waters on Starch Digestion

Temperature: 25° C. Amylopsin sol. contained 1mg. com'cl prep. per cc.

1	10cc 2% Starch	10cc 0.3% NaCl sol.	1/50cc Saliva	Achromic pt.	1.75 h
2	"	10cc dist. water	"	"	3.60 h
3	"	10cc soft (3-11)	"	"	5.75 h
4	"	10cc tap water	"	"	5.25 h
5	"	10cc soft (9-11)	"	"	9.25 h
6	"	10cc 0.3% NaCl sol.	1cc Amylop. sol.	"	1.00 h
7	"	10cc dist. water	"	"	2.25 h
8	"	10cc tap water	"	"	3.00 h
9	"	10cc soft (3-11)	"	"	3.00 h
10	"	10cc soft (9-11)	"	"	3.15 h

Table 16

Temperature 20° C.

1	50cc 2% Starch	50cc 0.3% NaCl sol.	5 cc Amylop. sol.	Time to the Achromic point	1.33 hours
2	"	50cc dist. water	"	2.80	"
3	"	50cc tap water	"	12.00	"
4	"	50cc soft (3-11)	"	12.00	"
5	"	50cc soft (9-11)	"	15.00	" #
6	"	50cc 0.3% NaCl sol.	1/10cc Saliva	1.80	"
7	"	50cc dist. water	"	4.33	"
8	"	50cc tap water	"	13.00	" #
9	"	50cc soft (3-11)	"	15.00	" #
10	"	50cc soft (9-11)	"	22.00	"

Tests marked # were estimated from color after 12 hours.

From series 15 and 16 it will be noted that the action of both salivary and pancreatic amylase was pronouncedly inhibited especially where the larger amounts of water were used,the order of inhibition being as before,recently softened water greatest and tap water least. Distilled water was a more satisfactory diluent whereas the sodium chloride solution was much more satisfactory than any of the waters mentioned.

Pancreatic amylase was somewhat less affected by the inhibiting agents than the salivary amylase probably because it naturally acts in a more alkaline medium.

It seemed quite probable that this inhibiting action of the waters softened with lime might be due to their greater alkalinity. Therefore both of these softened waters as well as tap water were titrated,first with methyl orage as an indicator to obtain the total amount of hydroxides and carbonates present,and second using phenolphthalein with boiling and cooling of the solution which gave the total hydroxides with one half of the alkaline carbonates not including any of the carbonates of the alkaline earths.
The results were as follows:

1. Total hydroxides and carbonates.

   Indicator: Methyl Orange

Tap water	required	3.3 cc N/5 HCl
Soft water(3-11)	"	1.8 "
Soft water(9-11)	"	1.8 "

2. Total hydroxides and one half of alkali carbonates

   Indicator: Phenolphthalein,with boiling and cooling.

Tap water	required	10.05 cc N/5 HCl
Soft water(3-11)	"	6.75 "
Soft water(9-11)	"	6.70 "

Test for Calcium:

10 cc of soft water(3-11) was acidified with acetic acid and ammonium oxalate solution added.On standing a few minutes a cloudiness due to calcium was produced. Tap water gave a much heavier precipitate.

It will be noted that the alkalinities of the two soft waters were exactly similar while that of tap water was somewhat greater.It appeared therefore that the differences of action could not be accounted for merely on the basis of alkalinity.

The calcium test was made to determine whether an excess of lime had been added.Only a slight test was however obtained such as might be accounted for by the presence of small amounts of calcium carbonate.A much more pronounced test was obtained with tap water.

In order to learn the composition of the softened water and to determine if possible the nature of the inhibiting factor Prof.E.Bartow,Director of the Illinois Water Survey,was consulted. From data submitted on the treatment of the University water supply with lime(51) it was determined that the following was the probable composition of the water used in our tests:

Sodium Carbonate	60.5	parts per	million
Calcium Carbonate (saturated solution)	30-50	"	"
Magnesium Hydroxide (saturated solution)	15-20	"	"

To determine the action of these substances separately and mixed on the enzyme hydrolysis of starch,the following solutions were made up:

Saturated solution of magnesium hydroxide(freshly made).

Saturated solution of calcium carbonate.

Solution of sodium carbonate 60 parts per million.

Saturated solution of magnesium hydroxide and calcium carbonate in sodium carbonate solution 60 parts per million.

The following preliminary test was made using in each case 50cc of 2% starch paste,0.1cc of saliva and 50cc of the water in question. The temperature was 20° C.

Table 18

Kind of Water	Time to the Achromic Point
0.3% NaCl sol.	4.00 hours
Distilled water	6.00 "
$CaCO_3$ sat.sol.	9.75 "
$Mg(OH)_2$ sat.sol.	10.75 "
Na_2CO_3 60 per million	12.15 "
Artificial softened water #	33.00 "
Softened water(9-11)	40.00 "

The artificial softened water is the above mentioned mixture containing 60 per million of Na_2CO_3 and being saturated with $CaCO_3$ and $Mg(OH)_2$.

It is probable that at the time of making this test the $Mg(OH)_2$ solution was not yet completely saturated as the inhibition is not so great as in later tests.

To determine more accurately the relations existing between these various waters ,and to find out if these ingredients were sufficient to account for the inhibiting action of the softened waters several series of quantitative tests were made.The first two series were run using the salivary enzyme and the second two using Armour's preparation of amylopsin.The reducing sugar was determined as usual by the reduction of Fehling's solution,

filtering off the resulting cuprous oxide, dissolving in nitric acid and titrating according to Kendall's modification of the copper-iodide method. The results are given in the following tables:

Table 19

Inhibition of the Action of Salivary Amylase by Certain Substances contained in Softened Water

Time of digestion: 20 minutes. Temperature: 35° C.

No.	Kind of Water	No.cc. Water	No.cc.of 4%Starch	No.cc. Saliva	No.cc.of $Na_2S_2O_3$	No.Mgs.of Maltose
1	0.3% NaCl sol	100cc.	25cc.	0.1cc	37.90	141.60
2	Distilled	"	"	"	7.95	31.80
3	$CaCO_3$ sat.sol	"	"	"	5.76	23.04
4	$Mg(OH)_2$sat.sol	"	"	"	0.59	2.36
5	Na_2CO_3 60p.m.	"	"	"	2.75	11.00
6	Artificial soft#	"	"	"	0.35	1.40
7	Tap water	"	"	"	0.85	3.40
8	Soft(3-11)	"	"	"	1.15	4.60
9	Soft(9-11)	"	"	"	0.23	0.92
10	Check	"	"	"	7.85	

See note table 18.

All columns corrected for check test.

Table 20

Similar to series 19, except: Time: 1 hour Temperature: 26° C.

No.	Kind of Water	No.cc. Water	No.cc.of 4% Starch	No.cc. Saliva	No.cc.of $Na_2S_2O_3$	No.Mgs.of Maltose
1	Distilled	100	25 cc	0.2	27.76	111.04
2	$CaCO_3$ sat.sol.	"	"	"	16.71	66.84
3	$Mg(OH)_2$ sat.sol	"	"	"	0.47	1.88
4	Na_2CO_3 60 p.m.	"	"	"	10.80	43.20
5	Artificial soft#	"	"	"	0.02	0.08
6	Tap water	"	"	"	8.96	35.84
7	Soft(3-11)	"	"	"	4.31	17.24
8	Soft(9-11)	"	"	"	0.86	3.44
9	Check	"	"	---	6.49	

See note table 18. All columns corrected for check test.

Table 21

Enzyme used: Pancreatic Amylase. Time: 2 h.at 25°, 1 h.at 40° C.

No.	Kind of Water	No.cc. Water	No.cc.of 4% Starch	Pancreatic Amylase	No.cc.of $Na_2S_2O_3$	No.Mgs. Maltose
1	0.3% NaCl sol	100cc	25cc	1 mg.	46.05	184.20
2	Distilled	"	"	"	11.40	45.60
3	$CaCO_3$ sat.sol	"	"	"	16.30	65.20
4	$Mg(OH)_2$sat.sol	"	"	"	0.38	1.52
5	Na_2CO_3 60 p.m.	"	"	"	8.76	35.04
6	Artificial soft	"	"	"	0.59	2.36
7	Tap Water	"	"	"	8.00	32.00
8	Soft(3-11)	"	"	"	1.31	5.24
9	Soft(9-11)	"	"	"	0.24	0.96

All columns corrected for control tests.

Table 22

On the Inhibiting Action of Substances Contained in Soft Waters

Enzyme used:Pancreatic Amylase Time: 2 hours.Temperature:38° C.

No.	Kind of Water	No.cc. Water	No.cc.of 4%Starch	Pancreatic Amylase	No.cc.of $Na_2S_2O_3$	No.Mgs. Maltose
1	0.3% NaCl sol	100cc	25cc	2.5 mg	57.71	230.84
2	Distilled	"	"	"	14.55	58.20
3	$CaCO_3$ sat,sol	"	"	"	17.45	69.80
4	$Mg(OH)_2$ sat.sol	"	"	"	0.60	2.40
5	Na_2CO_3 60 p.m.	"	"	"	15.35	61.40
6	Artificial soft#	"	"	"	0.55	2.20
7	Tap water	"	"	"	11.00	44.00
8	Soft(3-11)	"	"	"	5.55	22.20
9	Soft(9-11)	"	"	"	1.55	6.20
10	Check	"	"	---	7.50	
11	"	"	"	---	7.80	

See note table 18. All columns corrected for check tests.

Table 23

Inhibiting Action of Substances Contained in Softened Water

Enzyme used:Pancreatic Amylase Time 1 1/2 hours. Temp.: 38° C.

No.	Kind of Water	No.cc. Water	No.cc.of 4%Starch	Pancreatic Amylase	No.cc.of $Na_2S_2O_3$	Mgs.of Maltose
1a	$Mg(OH)_2$ sat.sol	100cc	25 cc	20 mg	5.58	22.32
1b	"	"	"	"	5.60	22.40
2a	Artificial soft	"	"	"	2.40	9.60
2b	"	"	"	"	2.45	9.80
3a	Soft water(9-11)	"	"	"	14.40	57.60
3b	"	"	"	"	12.95	51.80
4a	Check(dist.water)	"	"	---	7.36	
4b	"	"	"	---	7.45	

Conclusions as to the Ingredients of Tap and Softened Water which have an Inhibitory Influence on the Action of Amylases; being a review of tables 19,20,21,22,and 23

Under the conditions of these experiments any one of the ingredients of softened water causes an inhibition of the action of salivary amylase as compared with distilled water,the order of inhibition being: 1, $Mg(OH)_2$ saturated solution; 2,Na_2CO_3solution; 3,$CaCO_3$ saturated solution. The magnesium hydroxide exerted by far the greatest inhibition,the amount of reducing sugar produced in its presence either alone or in mixtures,being almost negligible except in series 23 where large amounts of pancreatic amylase were used and the digestions conducted for a somewhat longer time. In the salivary digestions calcium carbonate exerted a very noticeable inhibitory action.Sodium carbonate solution still further decreased the activity. The softened waters were not quite as detrimental in their action as the artificial as the artificial mixture,but the more recently softened water approached it.

Where pancreatic amylase was used we notice a few differences. The most marked of these is perhaps that the calcium carbonate solution gave noticeably better results than the distilled water,i.e. it did not inhibit the action but aided it. Also the sodium carbonate solution gave nearly or quite as good results as distilled water. Further tap water gave good results, and the softened waters showed somewhat less inhibition than in the case of salivary amylase. These facts are in accordance with the known conditions as to the reaction under which these enzymes normally work,and is further evidence against their being

identical.

These indications as to the individuality of the two enzymes are not necessarily opposed to the findings of Musculus and v.Mering(52) and the confirmatory work of Külz and Vogel,(53) these authors showing that the same products are obtained in both salivary and pancreatic digestion. Nasse(54) based his conclusions as to their identity on the similarity of their action with relation to sodium sulphate,sodium nitrate, ammonium and potassium chlorides,and other salts,while Wohlgemuth supports (77) this view with results from the study of some neutral salts,acids, amino-acids and bases. On the other hand Vernon(55) has shown that saliva acts much more slowly in the first few minutes of digestion than pancreatic amylase and points out differences in the action of several animal as well as plant amylases.

Of course if the two enzymes are identical they must act similarly under all conditions, and apparent differences in their action constitute stronger evidence than any observed similarities and until these differences are satisfactorily accounted for on some other basis,we can hardly do otherwise than consider the enzymes distinct.

All of these experiments pointed to the conclusion that the magnesium hydroxide was the principal inhibiting substance contained in these contained in these softened waters,altho in the case of saliva sodium carbonate also an unfavorable reaction. As to the degree of this inhibition it will be seen that under the conditions of these experiments the magnesium hydroxide content reduced reduced the enzyme activity to about 1/100 of its value in a 0.3% salt solution.

That the softened waters used in these tests should not give quite as great an inhibition as the artificial mixtures or the magnesium hydroxide alone is readily accounted for by the fact that for some time these waters had come more or less in contact with the air, so that the magnesium hydroxide with its great affinity for carbon dioxide had probably become partially transformed into the less injurious carbonate. This also probably accounts for the differences observed between the softened waters prepared at different times.

As to the physiological action of these softened waters it is difficult to say. There can be no question however that with regard to salivary digestion their action must be less favorable than that of a distilled water or one having a favorable electrolyte concentration.

It was of interest to know whether a like inhibition was exerted by the magnesium hydroxide solution and the softened water toward the enzyme pepsin. To determine this and at the same time to find out whether this inhibition was due to the Mg ion or not, the series of tests outlined below were carried out, different amounts of these solutions being added to pepsin-HCl digestion mixtures and the extent of the digestion as measured by the Mett method (56) taken as an index of the activity of the enzyme under the different conditions.

Table 24

Peptic Digestion in the Presence of Magnesium Chloride

No.	No.cc.of Pepsin-HCl	No.cc.of Dist.Water	Water Tested	Mm.Albumin Average	Activity
1	10 cc	10 cc	---	1.53	2.36
2	10 cc	10 cc	---		
3	"	5 cc	5cc $Mg(OH)_2$sat.	1.60	2.56
4	"	"	"		
5	"	---	10cc "	1.53	2.36
6	"	---	10cc "		
7	"	8 cc	2cc "	1.60	2.56
8	"	"	2cc "		
9	"	5 cc	5cc soft(9-11)	1.60	2.56
10	"	"	"		
11	"	---	10cc "	1.40	1.96
12	"	---	10cc "		
13	10cc 2%HCl	10cc	---	0.00	0.00

Digestions carried out 36 hours at 40° C.

Figures are averages for four Mett tubes in each case.

In contrast to their inhibition of the action of the amylolytic ferments,we find here in the case of peptic digestion that the magnesium hydroxide solution and the softened water have very little influence. In the case where 10cc. of the softened water was added the action was not quite as strong,but the difference is most probably due to the decrease in acidity of the mixture due to the sodium carbonate of the softened water.

As the magnesium ion seems to have no strong inhibiting action and as the alkalinity of the solution is not sufficient to account for the delay which the magnesium hydroxide causes in starch hydrolysis, the most plausible explanation of this

inhibition is that the enzyme combines in some way with the colloidal magnesium hydroxide,which might not be removed by ordinary filtration. Similar combinations of enzymes with metallic hydroxides have been pointed out by Euler(57) and the subject of the inhibition of enzyme activity thru adsorption has been treated quite at length by Hedin(58).

The presence of magnesium chloride has apparently no marked effect on enzyme activity,and the solutions in question would probably exert little influence on any stage of digestion except salivary digestion,unless,as is not impossible,some of these liquids should pass thru the stomach without coming in contact with the gastric juice. In such a case there might be expected some inhibition of intestinal digestion.

We must not push these suggestions too far. R.Berg(59) has endeavored to show by statistical studies that there is a paralellism between alkalescence of saliva,good teeth,and the hardness of the water drunk by the individual. He has also pointed out that soldiers from hard water regions have greater chest measurements than those from regions where softer water prevails. To suggest a direct causation here seems to be straining a point. On the other hand,from the standpoint of the greatest efficiency of the organism,we can hardly agree with the conclusions of Dr.Ide (60) that the influence of the character of the liquid ingested is negligible because compensation is provoked. In deciding as to the desirability of any drinking water,its influence on the digestive enzymes should certainly be considered.

We should expect these undesirable features of a lime softened water to be eliminated by the subsequent treatment with carbon dioxide which is sometimes practised.

Purification of Pancreatic Amylase

For some of our work it was desirable to have a somewhat purer amylase preparation than the ordinary.A number of methods of procedure have been suggested.The earliest was that of Cohnheim(61) who carried the enzyme down from a water solution with a precipitate of calcium phosphate and then washed the enzyme out again with water,finally throwing down from solution again with alcohol. Our slight experience with this method would suggest that it is wasteful on account of the difficulty of washing the precipitate free from enzyme. A similar criticism holds for Frankel and Hamburg's(62) procedure consisting of treatment with basic lead acetate,sterilization by filtration,and finally special fermentation to remove sugars and proteins. Attempts have been made by glycerol extraction methods,for instance by Seegen and Kratschner(63) and by Hufner(64) but without very good results. The more successful methods have had as a basis fractional precipitation with alcohol and dialysis to lower the ash content. Such are the methods of Lintner(65), Osborne(66),Jegorow(67) and Szilagi.(68) To this class also belongs the more recently proposed method of Sherman and Schlesinger(69) who claimed to have produced amylase preparations much stronger than any previously described. This latter method gave most promise of good results and was adopted. The new method proposed by the same workers for the determination of amylolytic activity was also used to test the strength of our preparation.

Method:

As a starting point Armours preparation of "Amylopsin" was used. Ten grams of this were rubbed up in a mortar with 500cc of 50% alcohol and filtered. This solution was then dialyzed

against 50% alcohol,the alcohol being replaced four different times,the total time of dialysis being 66 hours. The solution was then filtered again. It was now poured into a mixture of alcohol and ether containing as much ether as would remain in the mixture after the 50% alcohol solution of the enzyme was added. The mixture was allowed to stand for a short time . A yellowish,oily precipitate settled to the bottom of the vessel.The supernatant liquid was poured off and the precipitate dissolved in the smallest possible amount of distilled water. Iy was then poured with stirring into a large bulk of 95% alcohol. A white,flocculent precipitate was obtained which was filtered off on a hardened filter,washed with alcohol and ether and dried in a vaccuum dessicator over sulphuric acid.

Grayish white scales were obtained which responded to the biuret test and were soluble with some difficulty but completely in water.The preparation was very active,its exact activity being determined as described in the next paragraph.

Sherman,Kendall and Clark's method(44) was followed which in brief consists in allowing the enzyme to act on 100cc of 2% starch paste for exactly 30 minutes at a temperature of 40°, both starch paste and enzyme being at 40° C. at the time of mixing. When the time for digestion was finished the action was stopped by adding 50cc of Fehling's solution and then heating in a boiling water bath for 15 minutes and immediately filtering off the cuprous oxide on a Gooch crucible. Kendall's titration method was used for determining the copper.

The results of tests made on the original "Amylopsin" and on the purified preparation, are given in the following table.

Table 25

No.	Amylase tested	Mgs.of Amylase	No.cc.of $Na_2S_2O_3$	Mg.Cu.	Mgs.of Maltose	Activity
1	Original	1 mg	45.40	274.7	209.2	209.2
2	"	0.5mg	26.70	161.5	103.0	206.0
3	Purified	0.2mg	62.15	376.0	304.0	1520.0
4	"	0.1	36.50	220.8	158.7	1587.0
5	Check	---	8.20			

Maltose and activity columns are corrected for check.

This preparation was about one half as strong as the most active preparations of Sherman and co-workers,and 7 1/2 times as strong as the original preparation. A good yield was obtained,about 30% of the theoretical,and the method appears to be a very efficient one for the purification of amylase and probably other enzyme preparations as well. It could probably be adapted to the purification of salivary amylase,and might be expected to give better results than Cohnheim's method in which the chances for loss are greater.

As already pointed out,our preparation agreed with those of Sherman and Schlesinger in giving the biuret test.These authors also found that a dilute solution of their preparation gave a coagulum on boiling and the filtrate gave a biuret test for proteose.These findings substantiate Osborne's view that amylase is a compound of an albumin with a proteose(66). Wroblewski(70) claimed it to be related to the proteoses,obtaining from his best preparation leucin,tyrosin and arginin. Loew(71) considered it to be a protein,while Lintner(65) and Hufner(64) believed it to be an oxidation product of protein as analyses gave lower nitrogen values than for true protein,while at the same time the usual

protein reactions were obtained.

On the other hand the early work of Cohnheim(61) who concluded that his preparations of salivary amylase were not proteins as they did not give the usual reactions,is supported by the later work of Frankel and Hamburg(62) who could obtain no biuret or xanthoproteic reaction from their amylase preparations.

The question is still unsettled owing to the lack of a good index of purity for enzymes.

On the Reactivation of Human Saliva which has been made Inactive by the Action of Heat or of Acid,and including some Tests on the Activation of Dog Saliva

1 On the Reactivation of Heated Saliva

M.Roger showed that when 1 or 2 cc.of saliva,previously (72) heated to from 80 to 100° C.,thereby losing its activity,was added to a starch paste containing a drop of fresh saliva,a larger amount of starch was hydrolyzed in a given time than if this heated saliva was not added. He therefore presumed that an activation of the heated saliva had taken place. His results did not however appear to be conclusive ,as the effect produced by the comparatively large amounts of electrolyte which were added in this saliva,were not considered. As many of our own tests have shown a great difference in the amount of starch hydrolyzed,due to small differences in the electrolyte concentration,it seemed quite probable that the increases in Roger's tests were due to a similar cause. To prove absolutely that such is the case is difficult because the electrolytes of the saliva can be only approximately replaced,also the presence of the protein and other materials of the saliva may exert some effect. However if it appears beyond a reasonable doubt that the changes in electrolyte will account for the differences it should be unnecessary to look further.

The first series of tests were made as follows:

The heated saliva was prepared by heating in a test tube on the water bath at 85-90° C. for fifteen minutes. Twenty cubic centimeters of 1% starch paste were used in each case,the volumes being made the same in all cases by the addition of distilled

water. The activating solution was a 1:200 solution in distilled water so that the actual amount of fresh saliva added in each case where it was used was 1/200 of a cubic centimeter.

Table 26

No.	Amount of 1% Starch	Heated Saliva	Amount of Activating sol.	Time to the Achromic Point
1	20cc	----	1 cc	11.0 hours
2	"	1cc heated sal.	----	-----
3	"	"	1 cc	5.0 "
4	"	2cc 1% NaCl sol	"	5.5 "
5	"	3cc "	"	5.0 "
6	"	1ccHeated saliva 3cc 1% NaCl sol.	"	4.0 "

This series shows that the enzyme ,both before and after adding the heated saliva suffered from an electrolyte starvation, i.e. the lack of electrolyte limits its action. It also shows that the addition of sodium chloride gave as good results as the addition of the heated saliva. Somewhat more sodium chloride was however required to replace this saliva than average analyses suggest that it contains,and doubtless the action of other electrolytes which were not substituted entered in. The question becomes one mainly of how great the difference is and considering this we can see that the reactivation of even 1/100 of this heated saliva would have produced pronounced and unmistakeable differences in the time of digestion. Such differences were not found.

Another series of tests serves to corroborate this view. The following saliva solutions were used:

1. Fresh saliva.

2. Dialyzed saliva.

3. Fresh saliva heated 5 minutes at 80° C.

4. Dialyzed sal. " " "

5 Fresh saliva " 10 " "

6 Dialyzed sal. " 10 " "

7 Fresh saliva " 20 " at 100°C.

8 Dialyzed sal " 20 " "

Table 27

Activation of Heated Saliva

Activating solution :Fresh saliva 1:200

No.	Amount of 1% Starch	Heated Saliva	Amount of Activ.sol.	Total Vol. of solution	Time to the Achromic Point
1	10 cc	---	1 cc	15 cc	2.00 hours
2	"	0.3cc $NaCl, Na_2HPO_4$ sol.	"	"	1.50 "
3	"	1 cc of Saliva No.2	---	"	0.30 "
4	"	1 cc of Saliva No.3	---	"	-----
5	"	"	1 cc	"	1.75 "
6	"	1 cc of Saliva No.4	---	"	-----
7	"	"	1 cc	"	2.00 "
8	"	1 cc of Saliva No.5	---	"	-----
9	"	"	1 cc	"	1.75 "
10	"	1 cc of Saliva No.6	----	"	-----
11	"	"	1 cc	"	2.00 "
12	"	1 cc of Saliva No.7	-----	"	-----
13	"	"	1 cc	"	1.75 "
14	"	1cc of Saliva No.8	-----	"	-----
15	"	"	1 cc	"	2.50 "

Tests containing dialyzed saliva digested in about

the same time as those to which only the activating solution was added. Variation in the method of heating produced no effect either with dialyzed or heated saliva ,5 minutes at 80° C. being apparently as effective in destroying the action as 20 minutes at boiling. Tests containing heated fresh saliva digested in a slightly shorter time. That this was due to the electrolyte added is made probable by the fact that the addition of an approximate equivalent of sodium chloride in No.2 caused more rapid digestion than where the heated saliva was added. The greater rapidity of digestion here may be due to inhibitory substances produced on heating or the saliva may not have contained quite as much chloride as average analyses suggest. At any rate there was no sign of any reactivation of heated saliva.

In this connection the work of Walker(73) and of Gramenitzki(74) is of interest. Walker claims that ptyalin solutions inactivated by heating to 50-53° C. may be reactivated by blood. They are not reactivated if heated to boiling nor by previously heated blood. The author suggests the presence of a non-stable ptyalokinase and a more thermostable ptyalogen.

Gramenitzki endeavors to show that Taka-diastase solutions when heated to boiling for several minutes gradually regain a part of their activity on standing in the cold. Many of his digestions were carried on for several days and even months without antiseptics altho,he says,with special precautions against bacterial action. On whether these precautions were sufficient or not must depend the accuracy of the results,and this,thru lack of control tests we cannot decide with certainty. Taka-diastase is less readily affected by heat than salivary amylase,so that the work is not

directly comparable with ours. Walker's experiments are likewise not directly comparable on account of the low temperature of heating(50°C) employed. In general most authors agree that salivary amylase is destroyed at from 70-80° C.

2 On the Reactivation of Saliva which has Lost its Activity thru the Action of Natural or Artificial Gastric Juice

Roger(75) has found that if saliva is treated with an equal amount of gastric juice or of hydrochloric acid corresponding in strength to that of the gastric juice,after standing for some time it loses its activity;but if one or two cubic centimeters of this inactivated saliva is added to a starch paste and a drop of fresh saliva added,more reducing sugar is obtained than in similar tests carried on without the addition of this acidified and neutralized saliva. This Roger claims to be due to a reactivation of the saliva and on this basis he assumes that the saliva after being inactivated by the gastric juice of the stomach,is neutralized in the small intestines and reactivated either by traces of saliva which were not destroyed or by the pancreatic juice which he claims possesses a similar property. If it were true that the saliva thus continues to act in the intestines,this work is of considerable importance. As the experimental data brought forward in support of this seemed hardly sufficient for the drawing of such conclusions,and as we have not noticed any corroboration of this work it seemed worth while to make a few similar experiments.

In these tests we meet with the same difficulties that

were found in the work on heated saliva(not the difficulties of repeating Roger's work altho he gives no information as to how he determined the very small amounts of reducing sugar found in some of his tests) but in trying to take into account the electrolyte concentrations,a thing which he has entirely omitted to do. In the first place we know only approximately the amounts of various salts contained in the saliva and can not substitute them exactly,and secondly a difficulty was found in giving exactly the same alkalinity to two mixtures which do not act with the same sensitiveness to an indicator.Also it is evident that if the slightest trace of saliva should escape the action of the acid the results would be vitiated. A series of tests is given below.

The following solutions were prepared:

Series A

1. 5cc of saliva and 5 cc 1% HCl
2. " " 0.5% HCl
3 " " 0.25% HCl
4 " " 0.12% HCl

These mixtures were placed two hours at 40°,then made slightly alkaline to rosolic acid with N/80 KOH and each made up to 50cc.

Series B

1. 5cc of 1% Hcl
2. " 0.5% "
3 " 0.25% HCl
4 " 0.12% "

These were neutralized and made slightly alkaline to rosolic acid with N/80 KOH. They were then made up to 50cc.

The activating solution was a 1:100 solution of saliva.

Method: In each of 13 200cc flasks was introduced 50cc of a 2% starch paste,and to each was added 10cc of one of the solutions above. Then to all but the last five,which were used as checks, 1cc of the activating solution was added. To the second series to which none of the acidified saliva was added, 0.3cc of a solution of 0.3% NaCl and 0.02% Na_2HPO_4 were added to substitute in an approximate manner the electrolytes contained by this saliva. The digestion continued 1 hour and 40 minutes at 35° C.,at the end of which time 35 cc of Fehling's solution was added to each in order to stop the action,and the reducing sugar was determined in the usual manner.

Table 28

Reactivation of Acidified Saliva

					- Check	A minus B
1	10cc of	1A;	1cc Activ.sol	105.7mg Cu	92.1mg Cu	20.1
2	"	2A	"	215.0	77.5	- 3.7
3	"	3A	"	92.0	58.7	-10.9
4	"	4A	"	85.7	58.1	-10.0
5	"	1B	"	85.6	72.0	
6	"	2B	"	94.8	81.2	
7	"	3B	"	83.2	69.6	
8	"	4B	"	81.7	68.1	
9	"	--	---	13.6	00.0	
10	"	1B	---	13,6	00.0	
11	"	2B	---	134.5	123.9	
12	"	3B	---	33.3	19.7	
13	"	4B	---	27.6	14.0	

Tests 1A and 1B were repeated after acidifying and then making as nearly equally alkaline as possible. Both tests reached the achromic point at the same time,tending to show that the previous

variation was due to slight differences in the reactions of the mixtures.

The results as given by this series are not very satisfactory as they do not show sufficient concordance. This especially appears in No.1 which gave somewhat higher results in the presence of the treated saliva altho this discrepancy did not appear in the second test made to the achromic point with iodine. The reason,as stated is most probably that the degree of acidification was not exactly the same due to the difficulty of obtaining an exact end point with either rosolic acid or phenolphthalein in a mixture containing much saliva. The same difficulty was found in a second series carried out as above,the results again not being in concordance with each other. The data however,tho not very accurate,show at least that much less than 1/100 of the one cubic centimeter of treated saliva could have been reactivated,and consequently that such activation is of little if any practical import. Further careful work should be carried out.

Wohlgemuth(77) has also pointed out that neutralized gastric juice aids salivary digestion and credits this mainly to the sodium chloride formed,altho other thermostable substances may also exist which have the same influence.

Bierry(76) showed that to a certain extent gastric juice may also aid salivary digestion by reducing the alkalinity,saliva being most active at about 4/5 of its natural alkalinity.

Wohlgemuth's work on fecal amylases (34) is also indirect evidence against Roger's view of the reactivation of saliva in the intestine. It has been shown that in man occlusion of the pancreatic duct causes the amount of amylase in the feces to decrease to a

very small amount,so that the amylase of the feces can be taken as an index of the pancreatic function. This could not of course be the case were salivary amylase active in the intestine.

Some Tests on the Activation of Dog Saliva

The presence of a specific amylolytic ferment in the saliva of carnivora,particularly dogs,has long been an unsettled question. It now seems highly probable that such an enzyme does not exist. The older experiments of Bernard(4) and others in which the saliva was activated by long contact with the air are clearly misconceptions due to ignorance of bacterial action. The traces of amylase frequently reported for dog saliva are most probably,as Carlson(78) has pointed out due to blood and lymph diastases. The finding of sugar in the stomach after starch ingestion,as reported by Jacubowitch(79) and others and the failure to find such sugar in similar experiments by Bidder and Schmidt(80),are most readily accounted for,as has been suggested by Wohlgemuth(77) by a regurgitation of the intestinal contents. However,it is not impossible that the saliva might be activated either before or after it reaches the stomach,and thus take part in starch digestion.

The following tests on the activation of dog saliva were suggested by similar work carried out by Mr.Keeton in the Department of Physiology of this university.

The saliva used was obtained from dogs under ether anaesthesia. It was kept under toluol.

Table 29
Activation of Dog Saliva

Tests carried on in incubator at 40°C. Activator: Human Saliva

No.	No.cc.of 1% Starch	No.cc.of Dog Saliva	No.cc.of Human Saliva	Time to the Achromic Point
1	25 cc	---	---	---
2	"	---	0.5 cc	24 minutes
3	"	1 cc	---	---
4	"	1 cc	---	---
5	"	1 cc	0.5 cc	46 minutes
6	"	1 cc	0.5 cc	46 minutes

In these cases the saliva from two different dogs both delayed the digestion. One of these had stood under toluol for a day but that this did not affect the results was indicated by the fact that human saliva which had stood in a similar manner for two days showed no decrease in activity.

In the next series the action of dog saiva was compared with that of boiled human saliva.

Table 30

Activation of Dog Saliva

No.	Amount of 1% Starch	Amount of Dist.Water	Inactive Saliva	Amount of Human Saliva	Time to the Achromic Point
1	20 cc	5 cc	---	---	---
2	"	3 cc	2cc dog saliva	---	---
3	"	2 cc	"	1/400 cc	9 hours
4	"	3 cc	"	---	---
5	"	2 cc	"	1/400 cc	9 hours
6	"	4 cc	1cc boiled sal	---	---
7	"	3 cc	"	1/400 cc	11 hours

These tests were covered with toluol and incubated at 40° C.

The differences in time to reach the achromic point where dog saliva and boiled human saliva were used may be considered due to the greater electrolyte concentration of the dog saliva as well as to its greater amount as used in these tests. Dog saliva contains 2 to 4 times as much chlorides as human saliva(81).

That covering with toluol,without thoroly shaking the mixture with it,does not prevent the growth of bacteria was shown by the fact that all of the tubes in the last series gave an increased reduction after 48 hours,the largest amounts of course being found where the greatest amount of saliva gave the bacteria the best chance to grow. This would appear to be a fruitful source of error unless very especial precautions were taken.

Another series on dog saliva,in one case the electrolytes being approximately substituted by 1% NaCl.This amount was calculated from data given by Bottazi(81).

Table 31

Activation of Dog Saliva

No.	No.cc.of 1% Starch	No.cc.of Dog Saliva	Human Saliva	Time to the Achromic Point
1	20 cc	---	---	---
2	"	---	1/200 cc	10.0 hours
3	"	2cc (Tr)	---	---
4	"	"	1/200 cc	6.5 hours
5	"	2cc (13)	---	---
6	"	"	1/200 cc	4.5 hours
7	"	1.2cc 1% NaCl	"	4.5 hours

(Tr) and (13) refer to the two dogs whose saliva was tested.

As will be noted the digestion took about twice as long with 1/200 cc of human saliva alone as when 2 cc of dog saliva

were also added. But by adding the approximate analytical equivalent of the chlorides present in this saliva,the time of digestion was about the same. It would seem then that when such slight changes in electrolyte concentration so markedly affect the time of digestion,particular pains must be taken to maintain equal concentrations of these substances if any accurate deductions are to be made. In the case of salivary experiments this is impossible to do in more than an approximate manner,and any slight variations must be attributed to known probable causes rather than to the entrance of some new factor.

On the Iodine-Starch Reaction at Low Temperatures

In carrying out some salivary digestion tests in concentrated mixtures at 0° C.,considerable difficulty was found in reading the achromic point with iodine,due to the fact that when a drop of the mixture was added to three drops of dilute iodine solution on a test tab,a slight greenish yellow color was obtained,not very clearly distinguishable from the pale yellow of the iodine solution. This greenish color gradually turned thru the dextrin brown to yellow as the mixture warmed up,showing that this was not the true achromic point,with the possibility that the brownish color was due to a reversion ,or that the iodine reaction was inhibited at zero degrees. The first possibility,that of a slight reversion was tested out as follows:

In each of a series of six test tubes was introduced 5 cc.of a 10% starch paste and 5 cc. of saliva both being cooled to 0°C. They were then kept at 0° until the apparent achromic point was reached. Then they were placed at 40° for a few minutes. The color "struck back" as usual and the reducing sugar was then determined and compared with the checks which had not been warmed up. The progress of the digestion was followed in another set of checks so that those tubes were not disturbed in which the sugar was afterward determined.

Table 32

No.1	70 min.at 0° C ; 40 min.at 40°	327.3 mg. Cu reduced
No.2	" ----------	261.3 mg. Cu reduced
No.3	113 min.at 0° C ; 12 min.at 40°	306.9 mg. Cu reduced
No.4	" ----------	286.2 mg. Cu reduced

There was evidently no reversion in either case.

The Iodine Test in Freezing Mixtures

It was thot that probably the iodine-starch reaction was inhibited at the freezing point to some extent and to determine the validity of this belief several tests were made. To dilute starch paste (both fresh and hydrolyzed to the erythro-dextrin stage) small amounts of dilute iodine solution were added,all being previously cooled to 0°. Some were also placed in freezing mixtures and cooled to -20° C.

At 0° the mixtures were not,of course frozen,and the blue starch color and the reddish-brown dextrin color were given as usual.

In freezing mixtures at -20° C the following observations were made. Deep blue starch tests became reddish-violet. Light blue tests passed thru a violet to a very light orange pink. The deep violet-red dextrin color became a very dark brown. The light violet red of a more dilute solution passed thru brown to a brownish gray approaching black.

On warming the colors were restored to their former appearance,being however slightly less clear. In moderately concentrated solutions on standing in a warm room a heavy blue precipitate of the starch iodine compound settled out leaving a clear,colorless solution. The dextrin color was not lost so readily probably because being more truly in solution,even on being thrown out by freezing,on warming up again it redissolved. It is also probable that cooling to a still greater extent would entirely destroy the colors in these tests.

A Note on the Freezing of Starch Pastes

It is possible that the freezing method might be used as a means of separation of starches and dextrins,or of the constituents of the starch itself. For example 10.0cc. of a paste made from soluble starch and subjected for a moment to the action of saliva was frozen solid at -20° C. and then allowed to thaw out. On filtering,a solution was obtained giving the brown dextrin color with iodine but no starch test. The residue on the filter paper, on dissolving with the aid of heat gave a strong blue color with iodine. We were not however able to freeze out permanently all of the substance giving a blue color with iodine from a paste made from ordinary potatoe starch and not subjected to any hydrolysis. In these cases starch tests were always obtained in the filtrate altho hardened filters were used. This is in concordance with the views of Maquenne and Roux(82) and others,that starch contains an amylose soluble in cold water, very readily hydrolyzed and giving a blue color with iodine.

Malfitano and Moschkoff(83) have used a freezing method for the purification of starch,claiming that the electrolytes, which redissolve on thawing,are thus almost entirely removed,and a starch preparation obtained which gives pastes possessing a conductance not far from that of pure water.

Microscopical Observations on the Progress of Starch Digestion at 0° Centigrade

The original question of the cause of the reappearance of color with iodine in freezing digestion mixtures not being answered by the two previous series of experiments,it was thot that perhaps microscopical observations would elucidate the matter.

The 10% starch paste and the saliva used were filtered thru double hardened filters with suction. Five cc. of each were placed in a test tube and kept in an ice bath at 0° until only a slight greenish tinge remained on treating with iodine. Then the mixture was allowed to warm up slowly and at intervals one drop of the mixture with one drop of dilute iodine solution was placed on a slide and examined microscopically using the high power. The greenish tinge was found to be due to the absorption or enclosing of the starch by minute cellulose fibers and particles,partly from the original starch and partly perhaps from the filter paper. This enclosed paste stained blue with iodine and being protected by the cellulose wall was not digested by the amylase until the fibers themselves were disintegrated,this occuring very slightly if at all at 0° but being fairly rapid in strong saliva mixtures at room temperature. We have often observed that concentrated starch saliva mixtures placed at 0° will produce large amounts of reducing sugar without clearing up the solution,while at higher temperatures very little sugar is formed before the solution becomes clear. It has been suggested that there are two enzymes or enzyme activities concerned in starch hydrolysis,an amylase and a dextrinase and that the amylase is active at higher

temperatures than the other. If this be true it may be that conversely the dextrinase is comparatively more active at very low temperatures.

This two-enzyme theory of amylolytic activity has much support.The theory is based largely on the fact that on raising the temperature of the reaction mixture above a certain limit which varies somewhat with the particular enzyme and the time of exposure,the maltose forming power is almost entirely lost,while the starch liquefying power and dextrin forming power still remain. Among the authors who have noted and verified these phenomena are Brasse(84),Bourquelot(85),Horace,Brown and Glendinning(86),and Wijsman(87) who claims the support on this point of Märcker, Dubrunfaut,and Cuisinier.

M.Tschernowski gives the same view support by showing (88) that the two activities respond differently to nucleic acids. Wijsman followed the activity of amylases by means of the starch-iodine reaction in gelatin,and claims to have shown that there are two enzymes which possess different rates of diffusion in gelatin.

Frankel and Hamburg(62) claim to have made a fairly distinct separation of the two enzymes by dialysis,the dextrinase passing thru the membrane,while the amylase did not,there being apparently a difference in the size of the molecules.

Lintner from his extensive studies concludes,however,that there is but a single enzyme in the amylase of malt.(65)

Many of the phenomena mentioned above could just as easily be explained on the assumption of a different functioning of the same enzyme under different conditions or on the hypothesis of large enzyme molecules with side chains,these side chains possessing different properties. All can not be explained in this

way,however,and the two-enzyme theory seems to have the best support.

In connection with these experiments the work of E.T.Reichert(89) is of interest. He allowed pancreatin to act on a 1% starch paste until a violet reaction with iodine was given. The mixture was then quickly heated to 60-65° C. The blue color characteristic of starch appeared,this not being permanent unless the enzyme was destroyed. Mineral acid which destroys the enzyme does not prevent this return of color. It is concluded that the action is a dynamic and not an enzymic reversion. It seems possible ,however,that in these as in our experiments there is not a true reversion but merely a release of starch either mechanically or thru hydrolysis of the amylo-celluloses.

Brief Summary

1. The optimum dilution for salivary digestion lies at about four dilutions for a 0.3% sodium chloride solution,and at about seven dilutions for tap water and distilled water. The decrease in the rate of hydrolysis with greater dilutions is very marked with softened water,slightly less so with tap water,very gradual with distilled water,while with 0.3% sodium chloride solution the rate is very nearly constant for a great range of dilution.

2. Softened water,apparently on account of the magnesium hydroxide which it contains,strongly inhibits the action of the amylolytic ferments of saliva and the pancreatic juice,but not of pepsin in hydrochloric acid solution. On this account starch digestion proceeds less rapidly in softened water than in tap water and much less so than in distilled water or 0.3% sodium chloride solution. The action of sodium and calcium carbonate was also tested and the fact that the two amylases mentioned are influenced in different ways by these salts is evidence against their identity.

3. The methods of Sherman and Schlesinger for the purification of pancreatic amylase and the determination of amylolytic activity were found to give satisfactory results.

4. No reactivation of human saliva inactivated by the action of heat or of dilute hydrochloric acid,could be brot about by the addition of traces of fresh human saliva. These results are in disagreement with Roger's conclusions.

The dog saliva tested possessed no amylolytic power and could not be activated by traces of human saliva.

5. The starch-iodine and dextrin-iodine colors were found to be

markedly altered at temperatures from 0° to -20° C. The course of starch digestion is also somewhat changed at 0° C.

The possibility of a separation of starch from dextrins in digestion mixtures,by a freezing method,is suggested.

The writer wishes to thank Prof.P.B.Hawk,under whose direction these investigations were conducted,for his many helpful suggestions.

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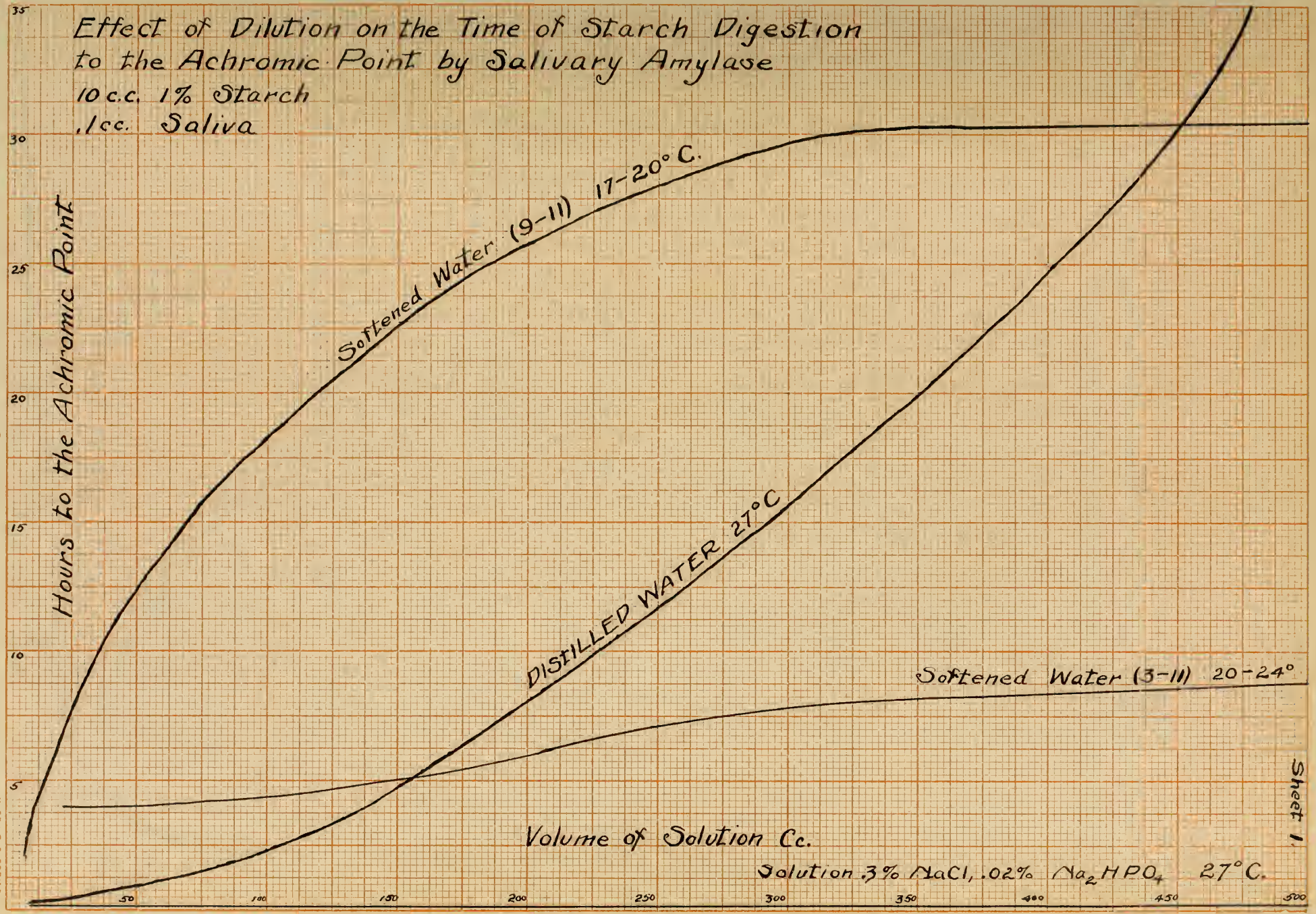
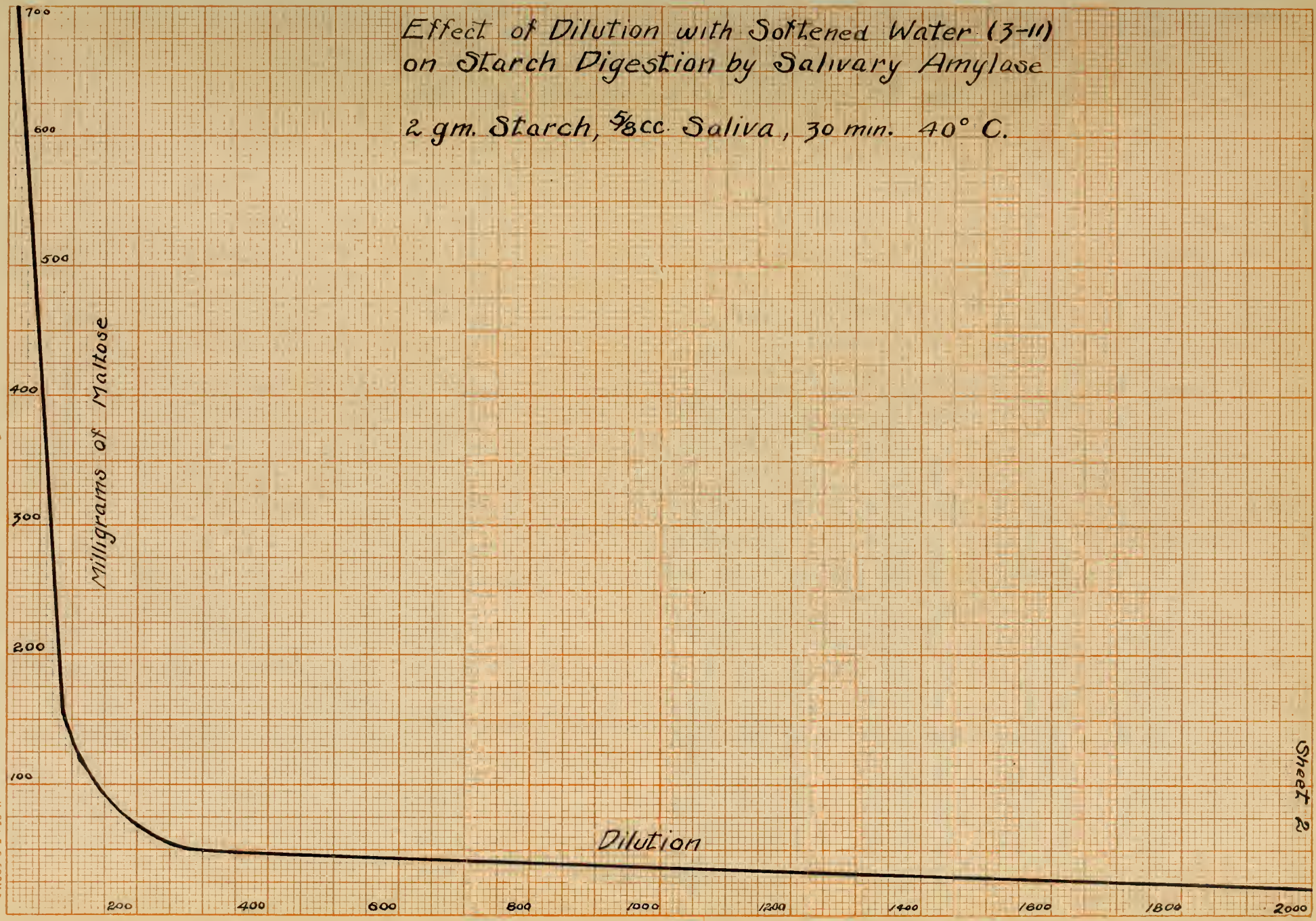
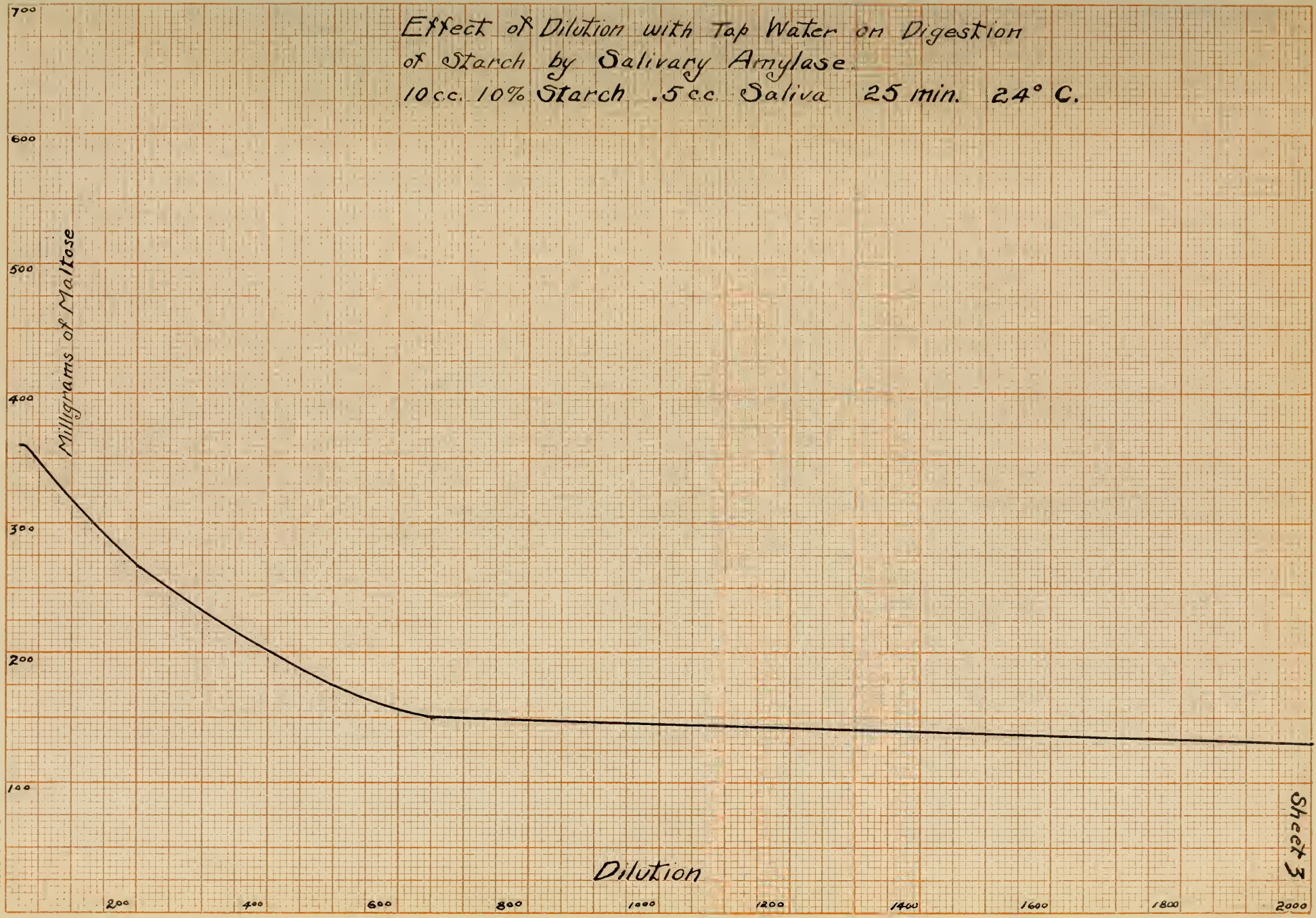
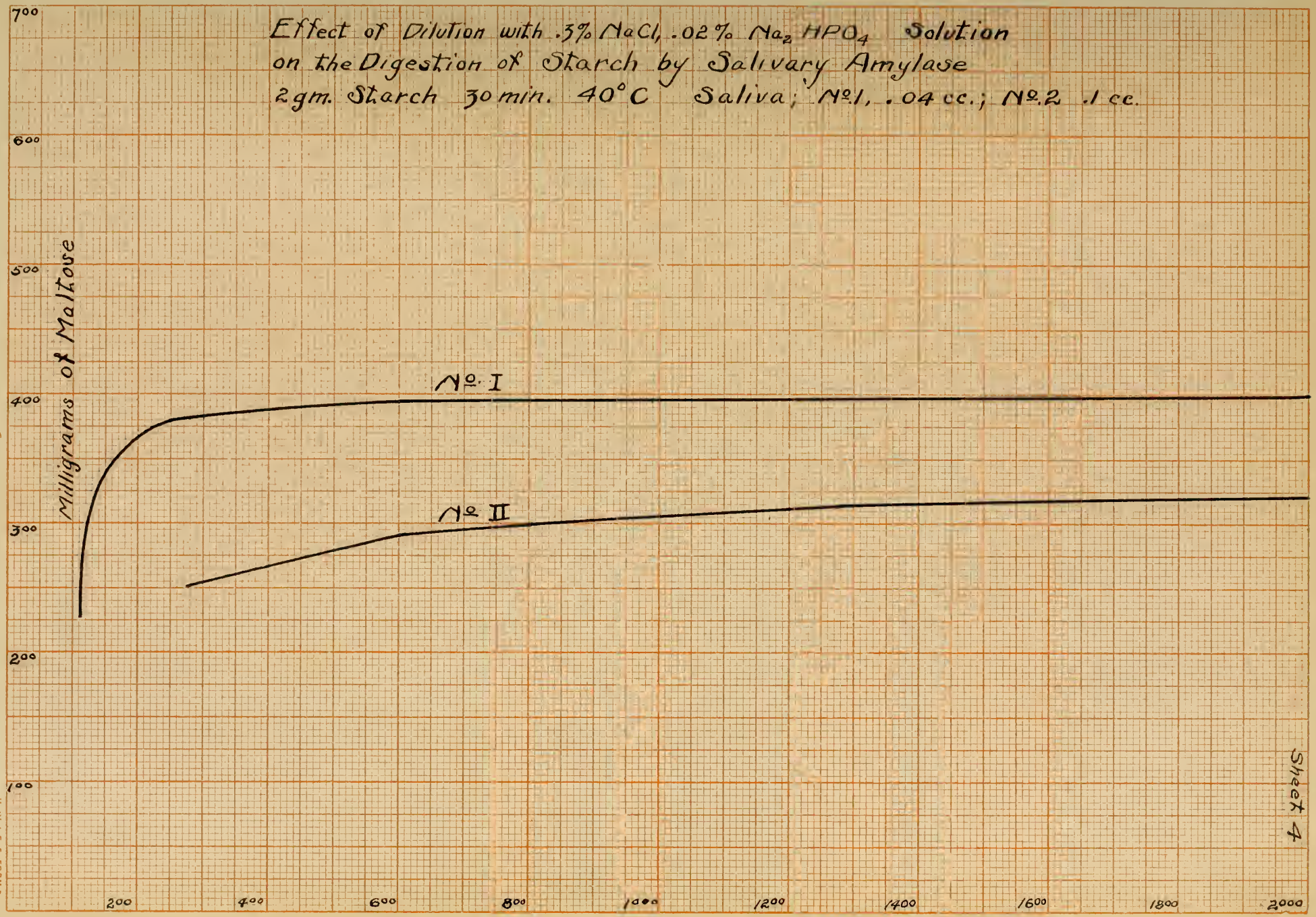

Effect of Dilution on the Time of Starch Digestion
to the Achromic Point by Salivary Amylase
10 c.c. 1% Starch
.1 cc. Saliva
Hours to the Achromic Point
35
30
25
20
15
10
5
Softened Water (9-11) 17-20° C.
DISTILLED WATER 27° C
Softened Water (3-11) 20-24°
Volume of Solution Cc.
50
100
150
200
250
300
350
400
450
500
Solution .3% NaCl, .02% Na2HPO4 27° C.
Sheet 1


Effect of Dilution with Softened Water (3-11)
on Starch Digestion by Salivary Amylase
2 gm. Starch, 5/8 cc. Saliva, 30 min. 40° C.
Milligrams of Maltose
700
600
500
400
300
200
100
Dilution
200
400
600
800
1000
1200
1400
1600
1800
2000
Sheet 2


Effect of Dilution with Tap Water on Digestion of Starch by Salivary Amylase.
10 c.c. 10% Starch .5 c.c. Saliva 25 min. 24° C.
Milligrams of Maltose
700
600
500
400
300
200
100
Dilution
200
400
600
800
1000
1200
1400
1600
1800
2000
Sheet 3
U. OF I. S. FORM 3

Effect of Dilution with .3% NaCl, .02% Na_2HPO_4 Solution on the Digestion of Starch by Salivary Amylase

2 gm. Starch 30 min. 40° C Saliva; №1, .04 cc.; №2 .1 cc.

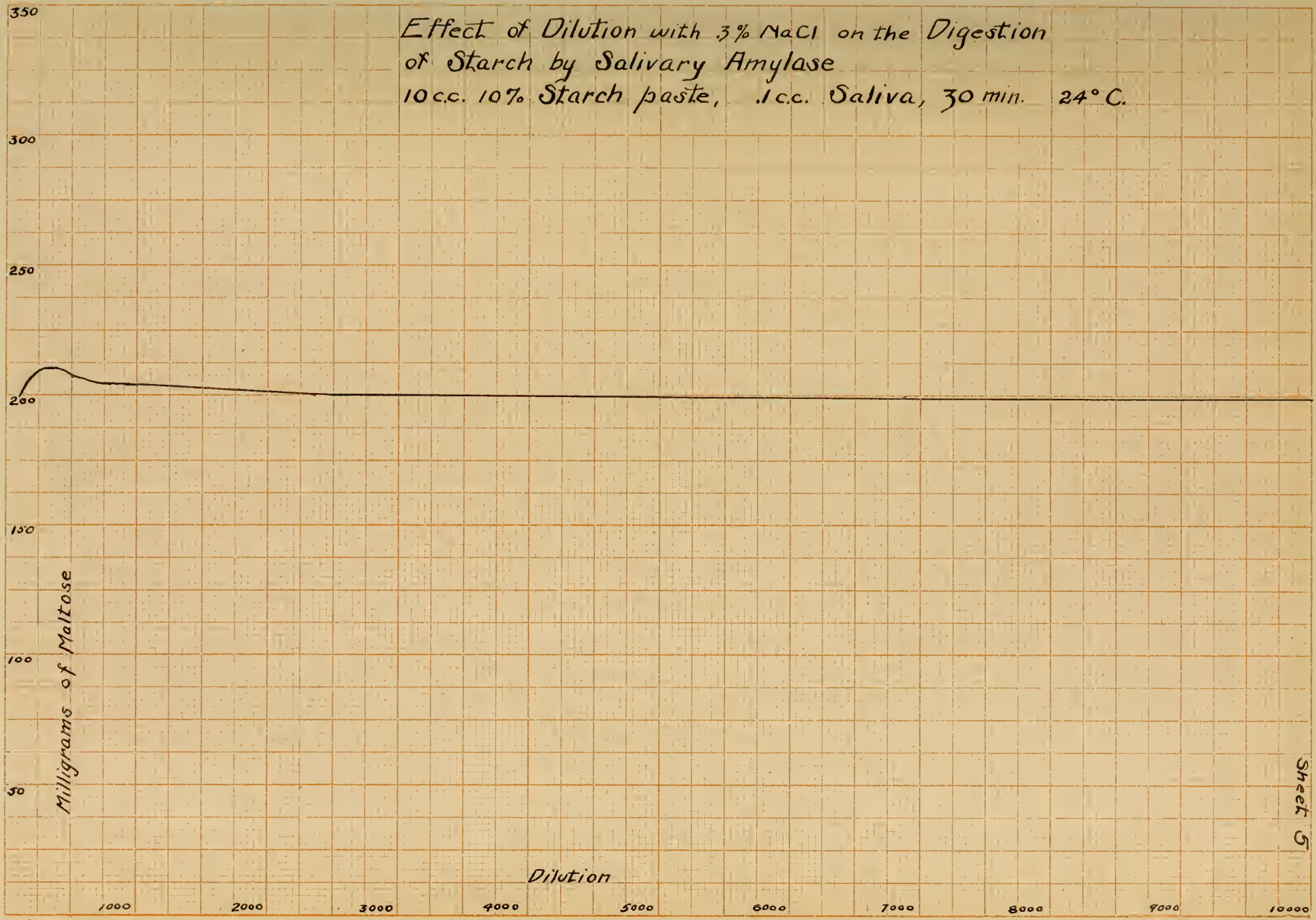


Sheet 4

U. U. I. S. FORM 1

Effect of Dilution with .3% NaCl on the Digestion of Starch by Salivary Amylase

10 c.c. 10% Starch paste, .1 c.c. Saliva, 30 min. 24° C.



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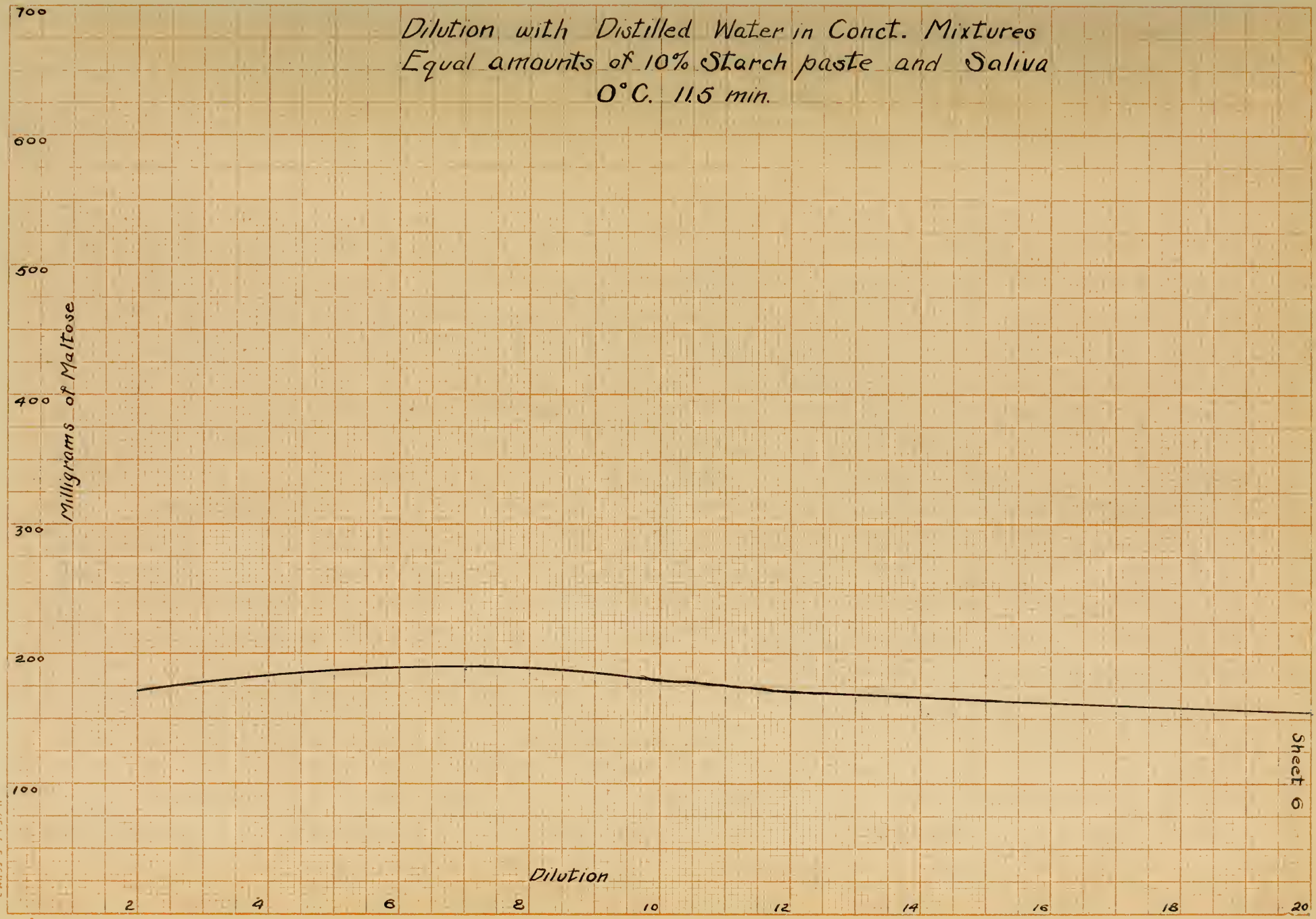
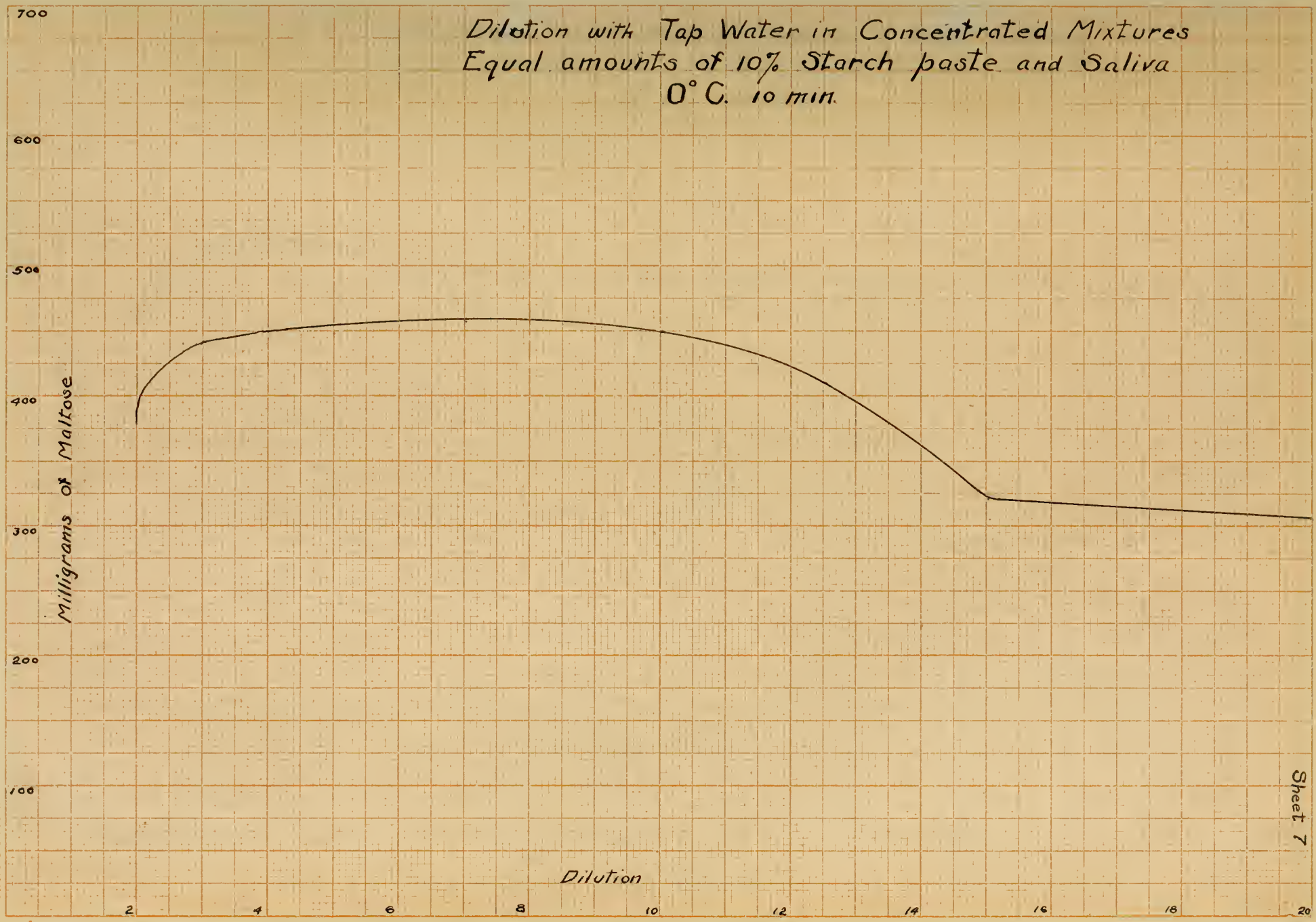

Dilution with Distilled Water in Conct. Mixtures
Equal amounts of 10% Starch paste and Saliva
0°C. 11.5 min.
Milligrams of Maltose
700
600
500
400
300
200
100
Dilution
2
4
6
8
10
12
14
16
18
20
Sheet 6


Dilution with Tap Water in Concentrated Mixtures
Equal amounts of 10% Starch paste and Saliva
0° C. 10 min.
700
600
500
400
300
200
100
Milligrams of Maltose
2
4
6
8
10
12
14
16
18
20
Dilution
Sheet 7


Dilution with .3% NaCl in Concentrated Mixtures
Equal amounts of 10% Starch paste and Saliva
0° C. 10 min.
Milligrams of Maltose
700
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
Dilution
Sheet 8